The control of chlorophyll formation and chloroplast development in the primary leaves of Phaseolus aureus.

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THE CONTROL OF CHLOROPHYLL FORMATION AND CHLOROPLAST DEVELOPMENT IN THE PRIMARY LEAVES OF *PHASEOLUS AUREUS*

Submitted by

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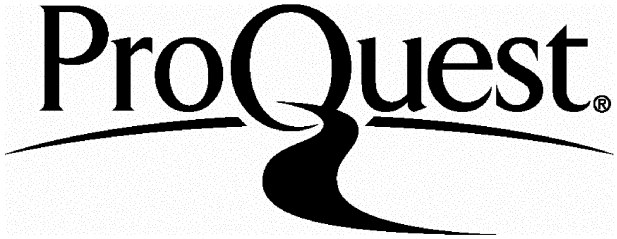

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ABBREVIATIONS

ALA	δ-aminolaevulinic acid
6-BAP	6-benzylaminopurine
B9 (CIBA)	N-dimethylamino succinamic acid
B9 (UNIROYAL)	N-dimethylamino succinamic acid
CCC	(2-chloroethyl) trimethylammonium chloride
CMU	(3-(4-chlorophenyl)-1:1-dimethylurea
DMF	Dimethyl formamide
DVA	γ δ-dioxovaleric acid
GA_3	Gibberellic acid
GA_4 ′ to GA_7	Other forms of gibberellin
IAA	Indoleacetic acid

SUMMARY

The effects of gibberellic acid and 6-benzylaminopurine on chlorophyll synthesis in the primary leaves of *Phaseoulus aureus* (mung bean) have been investigated. These have been related to the role of the cotyledon and hypocotyl in controlling chlorophyll formation during illumination of the etiolated seedlings.

The importance of the cotyledon to chlorophyll production declined with age after six days of etiolated growth and in five day old plants was of less significance than the presence of the hypocotyl. The presence of both organs resulted in interference and not an additive interaction.

Gibberellic acid and 6-benzylaminopurine promoted and inhibited chlorophyll synthesis. The nature of the response was dependent on the concentration of the hormone and the age of the leaf. In the absence of photosynthesis, the activity of both hormones was altered. Gibberellic acid proved ineffective and 6-benzylaminopurine inhibitory. The addition of sucrose to this or the control system (+ photosynthesis) indicated that gibberellic acid was more responsive to carbohydrate supply than was 6-benzylaminopurine. An examination of different carbohydrate sources revealed that sucrose most affected the hormone-induced responses.

The interaction of the two hormones showed a strong synergistic response in five day old leaves. This was present at

the higher concentrations of each hormone; the lower concentrations appeared antagonistic. Changes in the interaction were investigated with regard to age and substrate supply and were related to the level of endogenous gibberellin.

Possible effects of the hormones on chloroplast replication and carbon dioxide exchange were also examined.

The results are discussed in relation to the mode of action of the hormones and their role in the greening of primary leaves.

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INTRODUCTION

INTRODUCTION

1. Introductory Paragraph

Seedling growth through the soil or in imposed darkness is dependent on the available reserve stored within the seed. When the first leaves become illuminated they undergo many changes which transform them into assimilatory organs. The development of these leaves in the light is still very much affected by the presence of storage organs. Expansion of the primary leaves (Vyvyan, 1924; Wheeler, 1966) and chlorophyll synthesis (Wolff and Price, 1960; Sisler and Klein, 1963) in *Phaseolus vulgaris* are examples of this. Mung beans *(Phaseolus aureus)* are very similar to French beans *(P. vulgaris)* and it may therefore be concluded that observations on one species may be applicable to the other. The role of exogeneously applied substrates and hormones as a possible replacement for the cotyledons has been investigated (Wolff and Price, 1960; Sisler and Klein, 1963). No effects of hormone applications were observed, but other reports (see later) indicate that hormones do affect chlorophyll synthesis. The purpose of this work was to analyse more closely the inter-relationship of leaf chlorophyll synthesis and other organs and to examine the effects of hormones in conjunction with substrate supply.

2. Etiolation

Chlorophyll synthesis in most higher plants occurs only in the light in both etiolated and fully green material. Because of the extensive developmental changes which occur in light-treated etiolated plants, this affords an ideal system for the investigation of chloroplast development and its control.

The etiolated system is to some extent artificial, although this depends on the age of the plant material used. During normal light development the seedling experiences a degree of etiolation while growing through the soil and frequently the middle leaves of a closed apex are yellow. Usually one is experimenting with material which has been given an extended period of dark development. Under these conditions the normal features of etiolation (rigid stem elongation, lack of leaf expansion and absence of chlorophyll) are observed. The dark grown leaves contain characteristic plastids (etioplasts) which are about 3μ in diameter (Mego and Jagendorf, 1961) and contain a three-dimensional crystal lattice called the prolamellar body (von Wettstein, 1958). The etioplast develops from the proplastid which is a colourless, undifferentiated organelle approximately 1μ in diameter (from Kirk and Tilney-Bassett, 1967). In dark-grown barley, etioplast development took about 15 days (von Wettstein, 1958) and was accompanied by an increase in the formation of protochlorophyll (see Henningsen and Boynton, 1970). Since by this time the leaf under natural conditions would have experienced illumination, the formation of fully developed etioplasts would probably not have occurred. The etioplast is, therefore, a

dark-developed proplastid while a chloroplast is a light-developed proplastid. The extent of etioplast to chloroplast development which may occur under natural conditions will depend on the time it takes for the seedling to become exposed to light.

3. Protochlorophyll and Its Photoconversion

The existence of protochlorophyll was recorded as early as 1874 by Pringsheim and its role as the immediate precursor to chlorophyll was postulated by Monteverde (1893). The absorption spectrum was first discovered for a pure extraction of a non-phototransformable type obtained from pumpkin cotyledons. This corresponded well with the action spectrum for leaf chlorophyll formation (Frank, 1946) and was indentical with the absorption spectrum for purified leaf protochlorophyll (Koski and Smith, 1948). Final proof of Monteverde's suggestion was provided by Koski, French and Smith (1951) who correlated the peaks of the absorption and action spectra and by Koski (1950) who showed that for every molecule of chlorophyll formed one molecule of protochlorophyll disappeared.

The active form of protochlorophyll exists in association with a protein molecule and the complex is known as the protochlorophyll holochrome (Smith, 1960). The nature of the protein is not known. It has been suggested that it may be the enzyme ribulose diphosphate carboxylase (Trown, 1965) but distinctions between the holochrome and the latter have been made (Falk and

Bogorad, 1967). The stage of incorporation of protochlorophyll into the holochrome has been placed after the formation of the chlorophyll precursor (Granick, 1967). As will be described later (see Introduction 3) the pigment remains associated with the protein complex throughout a number of stages after photoconversion.

In etiolated leaves protochlorophyll exists in two forms, phytolated and non-phytolated (Loeffler, 1955). To avoid confusion the term protochlorophyllide ester is used to describe the former and protochlorophyllide, the latter. The use of the generic term, protochlorophyll is confined to instances where the form is not specified or both types are indicated (after Kirk and Tilney-Bassett, 1967). Evidence from absorption (Shibata, 1957) and fluorescence (Thorne, 1971a) spectrophotometry supported the presence of two forms of protochlorophyll. Only one of these, the longer wavelength-absorbing form protochlorophyll A650 was phototransformed (Shibata, 1957 ; Litvin and Krasnovsky, 1957 ; Sironval, Michel-Wolwertz and Madsen, 1965; Thorne, 1971a). Since protochlorophyllide is acidic while the ester is neutral it proved possible to separate them using polar and non-polar solvents. Wolff and Price (1957) exploited this difference and observed that it was the acidic protochlorophyllide which was phototransformed to chlorophyll. These results were confirmed by Virgin (1960). It may be concluded that the protochlorophyllide and protochlorophyll-A650 are the same molecule while the ester corresponds the form with an absorption peak at 636 nm. This is supported by Sironval *et al* (1965) who observed an increase in phytolation and the absorption peak with age in etiolated barley. Thorne (1971a) has shown that energy transfer

between the phototransferable protochlorophyll and previously synthesized chlorophyll molecules occurs and concluded that this comprises part of the holochrome while the non-phototransferable type does not. It is possible that protochlorophyll A636 may be converted to protochlorophyll A650 (Sundeqvist, 1969, 1970; Murray and Klein, 1971).

The conversion of protochlorophyllide to chlorophyllide is a photoreduction which takes place very rapidly, the rate being directly proportional to the light intensity (Smith and Benitez, 1954). The reaction was sufficiently resilient to withstand temperatures below $0^{o}C$, but ceased at $-195^{o}C$. The kinetics of the reaction suggested that it was not entirely photochemical, but involved intermolecular interactions (Smith and Benitez, 1954; Virgin, 1955). Any substance involved in the transformation must necessarily be very closely associated with the protochlorophyll molecule since at $0^{o}C$ the light induced absorption change is completed within 10^{-5} seconds, (Schopfer and Siegelman, 1969). The existence of different reducing systems in various species is exemplified by the loose relationship found in a mutant of *Arabidopsis* (Röbbelen, 1956). Protochlorophyllide could be reduced only in the presence of a soluble unpigmented fraction isolated from normal leaves. In some plants, however, such as conifers, ferns and mosses chlorophyll may be synthesized in darkness (Kirk and Tilney-Bassett, 1967).

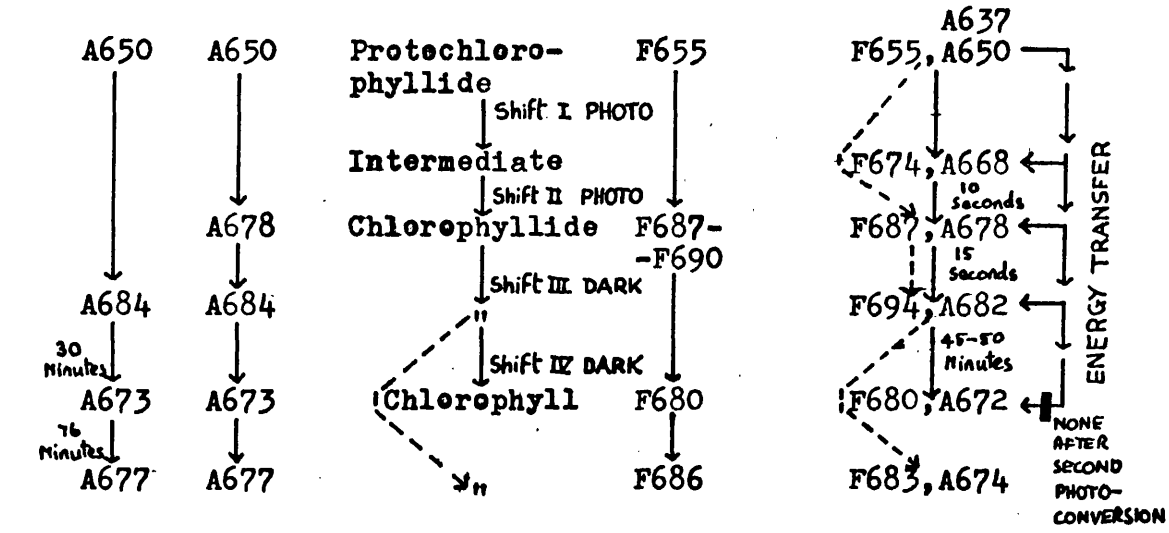
4. Changes In Chlorophyllide After Photoconversion

Concomitant with the reduction of protochlorophyllide to chlorophyllide there is a shift in the *in vivo* absorption maximum from 650 nm to 684 nm (Shibata, 1957). If the leaf is maintained in darkness, further spectral changes are observed. The sequence of spectral shifts has been studied by Shibata, 1957; Krasnovsky, 1960; Sironval, Michel-Wolwertz and Madsen, 1965; Gassman, Granick and Mauzerall, 1968; Bonner, 1969; Thorne, 1971a, 1971b). A scheme of the results is shown in Figure 1. The most recent work (Thorne, 1971a, 1971b) showed that the sequence changed when more than one flash of light was administered to the system. After a second photoconversion of protochlorophyll the fluorescence emission and absorption peaks of the final form of chlorophyll were different and there was no energy transfer between this form and protochlorophyllide. It was concluded that at this stage the chlorophyll molecule was removed from the holochrome structure.

The changes which result in the spectral shift from A684 to A673 are not known, but the latter has been correlated with phytolation (Vorobaeva, Bystrova and Krasnovsky, 1963; Sironval *et al*, 1965). Similar spectral shifts, however, can be induced by physical treatments such as freezing and thawing which result in disaggregation of the pigment molecules (Butler and Briggs, 1966). Akoyunoglou and Michalopoulos (1971) have reported that in bean leaves phytolation and this spectral shift are independent processes. It would seem, therefore, that any correlation is coincidental. The earlier spectral changes are probably a result of inter- and intramolecular changes within the pigment-holochrome complex.

FIGURE 1

SPECTRAL SHIFTS IN CHLOROPHYLLIDE AFTER PHOTOCONVERSION



Shibita (1957)

Shibita(1957)
Sironval et al.(1965)
Gassman et al.(1968)
Bonner(1969)

Krasnovsky(1960)

Thorne(1971a)
" (1971b)

KEY

——→ { Changes after initial photoconversion(Thorne's data)
{ Changes after illumination(other references)

- - - - - → Changes after second photoconversion(Thorne's data)

NOTE : Nomenclature and shift numbering after Thorne(1971a,1971b)

5. Time Course of Chlorophyll Synthesis

Following the initial rapid photoconversion of protochlorophyllide to chlorophyllide, there is a lag phase which may last for 1 - 2 hours and which precedes a phase of rapid chlorophyll synthesis (Koski, 1950; Virgin, 1955; Akoyunoglou and Argyroudi-Akoyunoglou, 1969). The lag phase is age dependent and is not apparent in young leaves (Sisler and Klein, 1963). In freshly excised leaves of *P. vulgaris*, which exhibited a 30 minute lag period, Wolff and Price found that this could be extended to 4 hours by preincubating the leaves on water in the dark for 18 hours. The addition of glucose or sucrose to the incubating medium during this period reduced the extended lag period as did the presence of one cotyledon attached to the leaf. Sisler and Klein (1963) observed that there was a rise in the capacity for chlorophyll synthesis between the 4th and 5th days of growth and concluded that some factor was exported from the cotyledons between these times. Removal of the leaves on the fourth day and then allowing them to develop to the fifth on sucrose was insufficient to produce the chlorophyll synthetic rate associated with 5-day old leaves. This agrees with the results of Wheeler (1966) for the effects of the cotyledons on the expansion of primary leaves in *P. vulgaris*. The possible role of growth regulators in this system is discussed in Section 14 of the Introduction. In addition to carbohydrates and the cotyledons, δ-ALA has been shown to shorten the lag phase in *P. vulgaris* leaves (Sisler and Klein, 1963), so it may be concluded that the lag phase is probably a feature of the shortage of substrate for chlorophyll synthesis.

In addition to chemical means, the lag phase may be overcome by a short impulse of red light followed by a dark period before illumination with white light (Withrow, Wolff and Price, 1956; Virgin, 1958). This effect was reversed by treatment with a short impulse of far red light after the red light (Withrow *et al*, 1956). The far red treatment was effective when given up to 9 hours after the red light although the response was reduced (Price and Klein, 1961). When alternating cycles of red and far red were used, the terminal quality of light determined the rate of chlorophyll synthesis during the exposure to white light (Price and Klein, 1961; Mitrakos, 1961). The length of the dark period between pre-irradiation and white light illumination is age dependent (Akoyunoglou, 1970) and is longer than the duration of the lag phase. The discrepancy between the two must be due to a continuous effect of light on chlorophyll synthesis other than on the photoconversion.

After the lag phase, chlorophyll is rapidly synthesized (Koski, 1950; Virgin, 1955; Akoyunoglou and Argyroudi-Akoyunoglou, 1969). This period correlates with the onset of grana formation (see Section 13 of Introduction) and the development of many of the attributes of mature chloroplasts.

6. Biosynthesis of Protochlorophyllide

Large quantities of protochlorophyll are formed when leaves are incubated on a medium containing ALA (Granick, 1963; Sisler and Klein, 1963). This observation and the similarity between the structures of chlorophyll and haem suggested that chlorophyll may be synthesized by the same biosynthetic pathway as haem. The biosynthetic sequence to the point at which the pathways diverge is shown in Figure 2. The details of this pathway have been elucidated from work on avian erythrocytes and mammalian liver. Direct evidence for the presence of this pathway in plants has been reported (Carell and Kahn, 1964; Rebeiz, Abou-Haider, Yaghi and Castelfranco, 1970). The results obtained by Carell and Kahn (1964) indicated that the pathway existed in the chloroplasts and that the chloroplast envelope exhibited differential permeability to certain of the precursors supplied.

The conversion of protophorphyrin IX, the compound which forms the branching point of the haem and chlorophyll pathways, to protochlorophyll involves the incorporation of magnesium and various alterations in the structure of the tetrapyrrole. The scheme for this part of the sequence is shown in Figure 3. The presence of several of the intermediates has been demonstrated in etiolated leaves treated with ALA (Granick, 1963; Rebeiz *et al*, 1970; Rebeiz and Castelfranco, 1971). The final conversion step of protochlorophyllide to chlorophyllide *a* has been demonstrated in isolated etioplasts on exposure to light (Klein and Poljakoff-Mayber, 1961). This step is followed by phytolation of chlorophyllide *a* to

FIGURE 2

COMMON BIOSYNTHETIC SEQUENCE OF PROTOCHLOROPHYLLIDE AND HAEM
(after Granick, 1963)

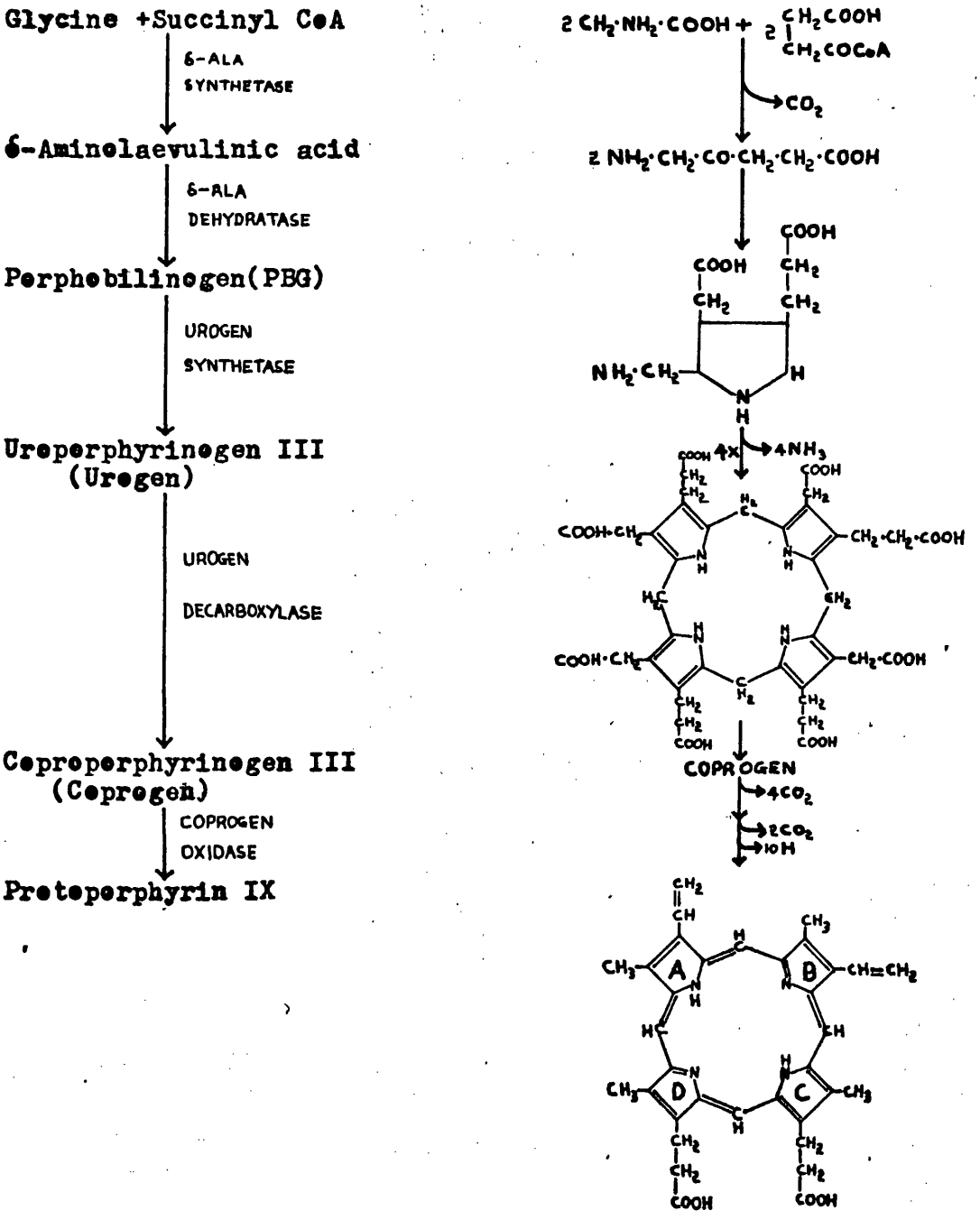
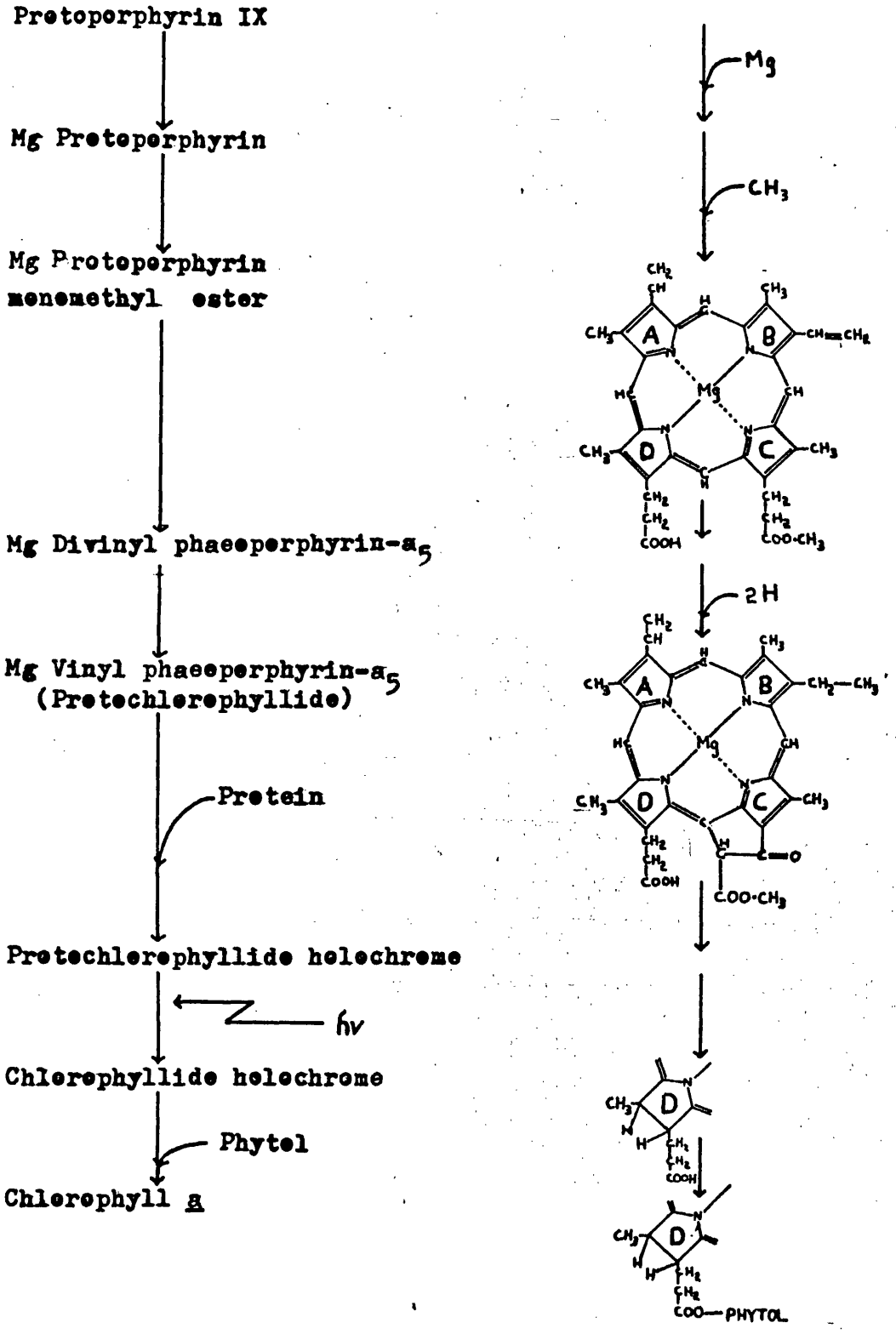


FIGURE 3

FORMATION OF CHLOROPHYLL *g* FROM PROTOPORPHYRIN IX
(after Granick, 1967)

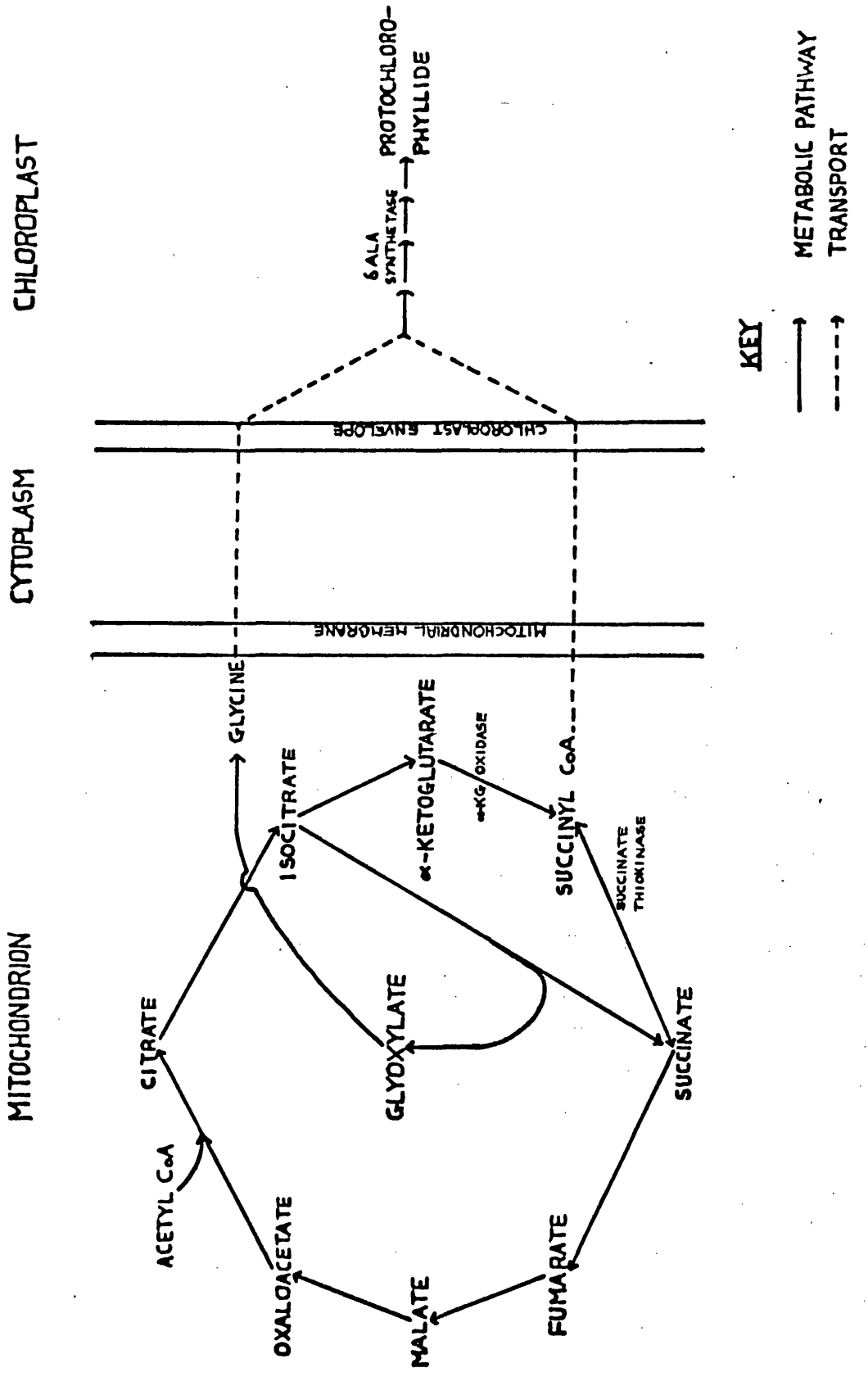


chlorophyll *a* which occurs during 20 - 25 minutes following illumination (Wolff and Price, 1957) depending on age (Akoyunoglou and Michalopoulos, 1971).

It would seem therefore that the chloroplast contains the means by which to synthesize chlorophyll from ALA. The subject of more recent discussions has been whether this latter compound represents the first step within the chloroplast (Kirk, 1970). The formation of δ-ALA is usually shown as being from succinyl CoA and glycine (Granick, 1963; Kirk and Tilney-Bassett, 1967), but the possibility of its production via the transamination of DVA also exists (Neuberger and Turner, 1963). The existence of this mechanism has not been reported in plants, although Hedley and Stoddart (1971a) have observed a close correlation between light-stimulated alanine amine transferase activity and chlorophyll synthesis. They suggested a close co-operation of this enzyme with δ-ALA amino-transferase, the former supplying substrates for the transamination of DVA to ALA. In a recent report Wellburn and Wellburn (1971) showed that isolated etioplasts were capable of incorporating radioactive succinyl CoA and glycine into chlorophyll *a*. This answers the original question of the position of ALA in chloroplast metabolism. Succinyl CoA and glycine are both produced in the mitochondria and are probably supplied to the chloroplast. Succinyl CoA synthetase has been found in bean (Steer and Gibbs, 1969), spinach (Kaufman and Alivisatos, 1955), tobacco (Bush, 1969) and wheat leaves (Nandi and Waygood, 1965) but does not reside within the chloroplast in barley leaves (Stobart and Pinfield, 1970). A summary of the supply of substrate to the plastid is given in Figure 4.

FIGURE 4

POSSIBLE SOURCE OF SUBSTRATE FOR PROTOCHLOROPHYLLIDE SYNTHESIS



7. Synthesis of Chlorophyll *b*

The appearance of chlorophyll *b* has been reported to occur 1 - 3 hours after the first observation of chlorophyll *a* in continuous white light (Seybold and Egle, 1938; Goodwin and Owens, 1947; Blaaw-Jansen, Komen and Thomas, 1950; Augustinussen, 1964; Butler, 1965; Oelze-Karow and Butler, 1971). Thorne and Boardman (1971) however recorded its appearance after only 10 minutes illumination and Shlyk (1971) concluded that chlorophyll *b* synthesis showed no delay, beginning almost immediately on illumination. It was, however, preceded by chlorophyll *a* synthesis. Whether chlorophyll *b* is formed from chlorophyll *a* or from protochlorophyll *b* is not known with certainty, but Shlyk (1971) concluded that the former mechanism operates. Its synthesis appears to be light controlled (Thorne and Boardman (1971) as exemplified by the fact that flashlight regimes favour chlorophyll *a* production (Akoyunoglou, Argyroudi-Akoyunoglou, Michel-Wolwertz and Sironval, 1966). Shlyk (1971) has suggested that newly formed chlorophyll *a* molecules are the precursors of chlorophyll *b*.

8. Development of Photosynthesis

The synthesis of chlorophyll is accompanied by the development of many other processes, one of which is photosynthesis. This process involves many factors which need not necessarily be formed simultaneously. The light trapping (electron transport) and carbon dioxide fixing systems may be conveniently separated and within each of these the formation of individual enzymes may be followed.

a) Formation of the Electron Transport System

Currently, this system is considered to comprise two light trapping centres which work in conjunction. One, photosystem II (PS II) oxidises water and produces oxygen, while the other, photosystem I (PS I) reduces NADP. The two systems are connected by an energy-releasing pathway which results in the formation of ATP. The process has recently been reviewed by Hall and Evans (1972).

The appearance of the oxygen-evolving capacity varies between species. It was first detected after 30 minutes in barley (Smith, 1954), 2 hours in oats (Blaauw-Jansen *et al*, 1950) and 16 hours in *P. vulgaris*. The development of this ability was not strictly correlated with chlorophyll synthesis as was evident from the results of Briggs (1920) and Smith (1954) which showed that after initial greening periods, oxygen-evolving ability was increased during subsequent dark periods.

Photosystem II activity as measured by reduction of Hill oxidants, is detectable at early stages of chloroplast development in barley leaves (Smith, French and Koski, 1952). Its detection lagged behind oxygen evolution and after 7 hours greening the rate of activity on a chlorophyll basis declined. A similar time-lapse before any PSII activity was detected, occurred in *P. vulgaris* leaves (Anderson and Boardman, 1964), and flax (Dodge and Whittingham, 1966). In contrast, Gyldenholm and Whatley (1968) first detected PSII activity in leaves of *P. vulgaris* between 10 and 15 hours.

Photosystem I activity is thought to precede the onset of PSII activity (Gyldenholm and Whatley, 1968); Plesnicar and Bendall, 1971; Odze-Karow and Butler, 1971). In peas this was not the case. Both activities began 4 hours after initial illumination (Dowdell and Dodge, 1971).

Although, dependent on the formation of chlorophyll, the photosystems are controlled by the synthesis of other factors some of which are light-independent (Oh-Hama, Shihira-Ishikawa and Hase, 1965) and others light controlled (Dodge and Whittingham, 1966; Dowdell and Dodge, 1971). The importance of light in controlling the development of the photosystems is emphasized by the preferential synthesis of PSI in flashlight regimes (Akoyunoglou and Argyroudi-Akoyunoglou, 1971). Transferring leaves from this regime to continuous light promoted PSII development (Michel-Wolwertz and Sironval, 1971; Akoyunoglou and Argyroudi-Akoyungoglou, 1971).

The formation of cofactors and enzymes involved in the photosystem has not been extensively investigated. Many have been

found to increase during greening (Dodge and Whittingham, 1966; Plesnicar and Bendall, 1971; Phung Nhu Hung, Remy and Moyse, 1971) but as yet none appear to have been specifically indicated as being limiting factors in the process of photosynthetic development.

b) Development of Carbon Dioxide Fixation

The onset of light-stimulated carbon dioxide uptake may be considered to mark the beginning of maturity for the chloroplast, since it must involve co-operation of a fully developed electron transport system and the Calvin-Benson cycle. It has been detected after 5 hours of illumination of wheat leaves (Tolbert and Gailey, 1955), 6 hours in *Euglena* (Stern, Schiff and Epstein, 1964) and 3 hours in barley (Rhodes and Yemm, 1966) and peas (Dowdell and Dodge, 1971). In etiolated bean leaves it was not observed until after 16 hours illumination (Bradbeer, 1969). This correlated with the time-lapse observed for oxygen evolution and the formation of the partial activities of the chloroplast (Gyldenholm and Whatley, 1968). The times observed for detection of carbon dioxide uptake were not dissimilar for those of oxygen evolution. In general, chlorophyll was not a limiting factor. As with oxygen evolution, carbon dioxide uptake increases during the dark after an intial illumination period (Gabrielsen, Madsen and Vejlby, 1961).

As might be anticipated, enzymes involved in photosynthesis increase in activity during greening. Ribulose diphosphate (Ru DP) carboxylase, phosphoribulokinase and triose-phosphate dehydrogenase (NADP) have been the subjects of particular interest (Chen, McMahon and Bogorad, 1967; Filner and Klein, 1968; Bradbeer, 1969).

These three are confined to the chloroplast but others which are present in both chloroplast and cytoplasm have also been observed to increase (Filner and Klein, 1968) as a result of light-stimulation.

The development of RuDP carboxylase has been correlated with chlorophyll synthesis (Dassiou and Akoyunoglou, 1969; Huffaker, Obendorf, Keller and Kleinkopf, 1966; Obendorf and Huffaker, 1970), but this is probably indirect since other evidence is available which demonstrates the development of the enzyme in the absence or at low levels of chlorophyll (Filner and Klein, 1968; Benedict and Kohel, 1969; Huffaker, Cox, Kleinkopf and Stanford, 1970). The appearance of the enzyme, triose-phosphate dehydrogenase (NADP) may be dependent on photosynthetic electron transport producing NADP (Ziegler and Ziegler, 1965). In bean leaves the lag phase of 15 hours (Bradbeer, 1969) prior to its induction, correlated closely with the appearance of photosynthetic activity.

Since the enzymes involved in carbon dioxide fixation are not as intimately associated with chlorophyll are those concerned with electron transport, a lesser dependence on chlorophyll synthesis may have been expected. Nevertheless, the operation of the system as a whole is controlled by each individual factor. Once photosynthetic competence is attained, the chloroplast is capable of supplying some of its own substrate. This aspect will be considered in Section 14 of the Introduction.

9. Protein Synthesis

Illumination of etiolated plants results in large increases in the amount of protein (De Deken-Grensen, 1954; Brawerman and Chargaff, 1959; Mego and Jagendorf, 1961). The plastid fraction has been indicated as being largely responsible for the increases (Rhodes and Yemm, 1963). General increases in total protein have also been recorded in connection with changes in individual proteins, (Filner and Klein, 1968; Bradbeer, 1969). Huffaker *et al* (1966) observed similar increases in particular enzymes but no change in total protein.

There is evidence to suggest that some of the proteins formed are synthesized within the chloroplast (Margulies, 1964; Smillie, Graham, Dwyer, Grieve and Tobin, 1967; Ireland and Bradbeer, 1971; Goodenough, Togasaki, Paszewski and Levine, 1971). Much of this evidence has been gained from the use of chloramphenicol which inhibits the activity of chloroplast ribosomes and cyclohexamide which interferes with cytoplasmic ribosomes (Kirk, 1970).

The relationship of chlorophyll synthesis and protein synthesis is considered in Section 14.

10. Nucleic Acid Synthesis

The direction of protein synthesis is ultimately dependent on nucleic acid synthesis. The point of interest is again whether the centre of control is the cytoplasm or chloroplast. Chloroplasts contain RNA (Smillie, 1963) and this increases during illumination of etiolated material (Rhodes and Yemm, 1963; Gyldenholm, 1968; Pollack and Davies, 1970; Poulson and Beevers, 1970; Smith, Stewart and Berry, 1970). Studies on the incorporation of radioactive precursors have indicated that chloroplasts are capable of synthesizing RNA (Bogorad, 1965; Spencer and Whitfield, 1967a; Bottomley, 1970). This evidence suggests that chloroplasts may also direct the synthesis of their own protein. They are also capable of synthesizing DNA (Spencer and Whitfield, 1967b; Kirk, 1970). During greening of etiolated bean leaves (Gyldenholm, 1968) no changes in DNA level were observed. Similarly Rhodes and Yemm (1963) found no differences between light and dark-grown barley. Conversely, in both spinach (Possingham and Saurer, 1969) and tobacco (Boasson and Laetsch, 1969) the number of plastids (and presumably therefore the total amount of plastid DNA) increased. The importance of chloroplast replication during greening is an area which does not appear to have been fully investigated.

The relative control of chloroplast metabolism by nuclear and chloroplast genes has been investigated using various mutants of barley (von Wettstein, Henningsen, Boynton, Kannangara and Nielsen, 1971) and *Chlamydomonas* (Goodenough *et al*, 1971). The results have indicated that many of the processes are controlled by nuclear genes.

II. Terpenoid Synthesis

Illuminated etiolated seedlings from large amounts of carotenoid pigments in addition to chlorophyll (Blaaw-Jansen *et al*, 1950). The function of these is uncertain but they may protect the chlorophyll molecule from photo-oxidation (Anderson and Robertson, 1960). The synthesis of carotenoids shares a common pathway with the synthesis of the phytol portion of the chlorophyll molecule. Terpenoid formation may be induced by a short impulse of red light (Cohen and Goodwin, 1962) and subsequently continues in the dark. The action spectrum for carotenogenesis is similar to the proto-chlorophyll absorption spectrum (Wolken and Mellon, 1956). This implies a close relationship between chlorophyll synthesis and light-induced carotenoid synthesis.

The control of terpenoid biosynthesis has been investigated by Goodwin (1958a, 1958b) and Mercer and Goodwin (1962, 1963). They have proposed that segregation of the enzymes within the cell and specific impermeability of the intracellular membranes are the principal components of control. More recent evidence on the localization of certain of the enzymes involved supports this theory (Rogers, Shah and Goodwin, 1966a, 1966b). The mechanisms of control involved in this area of synthesis may be applicable to other areas of chloroplast metabolism. The implications of selective permeability of the chloroplast envelope are discussed in Section 14.

The pathway of terpenoid biosynthesis is also responsible for the formation of gibberellins. The possible role of these in

chloroplast metabolism forms a large part of this thesis. In proposing a role for endogenously synthesized gibberellin, the source and formation of the hormone are important factors.

12. Lipid Synthesis

Unit membranes comprise both lipid and protein layers. The lamellar system of the chloroplast is no exception and consequently both protein and lipid are required for its formation. The prolammellar body is also made from these compounds and is known to give rise to the thylakoids formed during illumination. Gunning and Jagoe (1967) concluded that by the end of the lag phase no large areas of new membrane had been formed, which suggested that the membranous system at this stage had been formed largely by rearrangement of the prolamellar body. The necessity for lipid synthesis would, therefore, appear to be minimal except for qualitative changes which may occur. Increases in fatty acid (Crombie, 1958) and lipid content (Leech, Rumsby, Thomson and Crosby, 1971; Tevini, 1971; Trémolières and Lepage, 1971; Kannangara, Henningsen, Stumpf, Appelqvist and von Wettstein, 1971) during greening have been recorded. The production of linolenic acid was the most significant compositional change (Crombie, 1958; Wallace and Newman, 1965; Leech *et al*, 1971; Trémolières and Lepage, 1971). In peas a considerable contribution of fatty acids or precursors was made to the greening leaves by the cotyledons.

The plastid does posses the capacity for lipid biosynthesis but this is absent in young leaves (Kannangara *et al*, 1971). The level of activity and destination of labelled precursor were also modified by light. Givan and Leech (1971) have found that chloroplasts are capable of synthesizing some fatty acids but not linolenic acid, which is the major component formed during greening. Current evidence therefore indicates that the developing chloroplast is dependent on cytoplasmic synthesis of some of its lipid components.

13. Ultrastructural Development

After illumination the prolamellar body of the etioplast is gradually transformed into granal lamellae of the mature chloroplast. The process may be divided into three phases: tube transformation, vesicle dispersal and grana formation (Virgin, Kahn and von Wettstein, 1963). Tube transformation describes the period when the para-crystalline structure of the prolamellar body is changed into small tubular vesicles. It occurs at very low light intensities (Eriksson, Kahn, Walles and von Wettstein, 1961) and continues in the dark once stimulated (Henningsen and Boynton, 1970). The action spectrum for this process (Virgin *et al*, 1963) is consistent with the suggestion that protochlorophyll is the photoreceptor for the transformation. Whether the structural change is an obligatory consequence of proto-chlorophyll photoconversion or not is uncertain (Kirk and Tilney-Bassett, 1967) but there is a very intimate relationship between the prolamellar body and protochlorophyll synthesis. (Henningsen and Boynton, 1970).

The tubular vesicles gradually disperse to form double-membraned perforated sheets which align almost parallel with one another in the stroma (Gunning and Jagoe, 1965). This second phase also takes place at low light intensities (Eilam and Klein, 1962). The rate of extension is proportional to light intensity (Virgin *et al*, 1963),being completed within a few minutes at sufficiently high intensity. The photo-receptor which is not yet known, must be able to absorb the light (450nm) which is the only waveband which will stimulate the process (Henningsen, 1967).

Grana formation begins immediately after the lag phase of chlorophyll synthesis has elapsed and is directly correlated with the rapid phase of chlorophyll formation (Virgin *et al*, (1963). This correlation is not a direct one. The addition of CMU to greening reduces chlorophyll synthesis to a greater extent than it does grana formation (Klein and Neuman, 1966) and δ-ALA which promotes chlorophyll synthesis reduces the number of grana present (Klein and Bogorad, 1964). The addition of sucrose reversed the effect of CMU on grana formation indicating that the role of photosynthesis was not specific. In this treatment the grana stacks were larger and contained more thylakoids than in the controls (Klein and Neuman, 1966).

The ultrastructural organisation of the chloroplast represents the integrated form of the individual developing processes and is a demonstration of the enormous complexity of control systems required to transform an etioplast into a chloroplast.

14. Control

The development of photosynthetic capabilities is dependent on the synthesis of chlorophyll during the early stages of greening (See Section 8 of the Introduction) although the relationship is not necessarily a direct one. During these first few hours the synthesis of chlorophyll does not appear to be dependent on photochemical activity. Incubation of mung bean leaves on CMU showed that there was little effect on the chlorophyll level until more than 10 hours had elapsed (Dodge, Alexander and Blackwood, 1971). This is a few hours after the onset of carbon dioxide fixation (see "Results"), which indicates that there is a lag period before photosynthesis begins to contribute to chloroplast development. During the later course of chlorophyll synthesis, photosynthetic competence is a necessary factor (Klein and Neuman, 1966; Dodge *et al*, 1971). The effect of photosynthesis is non-specific as indicated by its replacement with sucrose (Klein and Neuman, 1966; Dodge *et al*, 1971). It has also been suggested that photosystem I may contribute substrate to the chloroplast by *in vivo* operation of cyclic phosphorylation in leaves treated with CMU and sucrose. This process may influence the uptake and utilization of sucrose. (Dodge *et al*, 1971). Photosynthesis may also contribute to chloroplast development by inducing the activity or synthesis of enzymes such as ribose-5-phosphate isomerase (McMahon and Bogorad, 1967) and NADP-dependent triose phosphate dehydrogenase (Ziegler and Ziegler, 1965). This mechanism was suggested as a possibility in peas (Dowdell and Dodge, 1970). Leaves greened at low light intensity for 48 hours possessed

inhibited carbon dioxide uptake when compared with leaves greened at high light intensity to the same chlorophyll level.

The use of inhibitors of protein synthesis has shown that chlorophyll synthesis is dependent on protein formation during greening. Chloramphenicol inhibits greening in *E. gracilis* (Pogo and Pogo, 1965), *P. vulgaris* (Margulies, 1967; Ireland and Bradbeer, 1971) and *Pisum sativum* (Dowdell and Doge, 1971). It may therefore be interpreted that chlorophyll synthesis is controlled by the synthesis of proteins. Many associated developmental processes, such as the formation of lamellae (Margulies, 1966), lipids (Bishop and Smillie, 1970) and photosynthetic capabilities are also diminished by chloramphenicol, thus chlorophyll synthesis may be inhibited indirectly by its effect on these other phenomena. Gassman and Bogorad (1967) have shown that the chloramphenicol inhibition during the lag phase can be partially relieved by the addition of δ-ALA and concluded that light regulated chlorophyll synthesis by controlling the availability of δ-ALA. This may have been mediated through an enzyme of δ-ALA synthesis. Since all the enzymes of protochlorophyllide synthesis from δ-ALA are believed to be present in the etiolated leaf (See Section 6 of Introduction) it is most likely that ALA synthetase is the enzyme involved (Kirk and Tilney-Bassett, 1967).

At a nucleic acid level it has been shown that chlorophyll synthesis is dependent on RNA synthesis (Bogorad and Jacobsen, 1964; Poulson and Beevers, 1970; Pollack and Davies, 1970) but that DNA synthesis was not an important factor (Smillie, 1963). This latter

observation conflicts with evidence which suggests that phases of rapid chlorophyll synthesis correlate with chloroplast division (Boasson, Laetsch and Price, 1972). The direction of RNA synthesis in the chloroplast may come from nucleic and chloroplast DNA. This aspect is an area of active interest at the present (Kirk, 1970).

The developmental interaction of chloroplasts and cytoplasm may be carried further by consideration of the substrates required by the chloroplast. During the early stages of development, photosynthesis is absent and many other syntheses are at a low level. During this period the chloroplast is almost entirely dependent on the cytoplasm for carbohydrates, amino acids, fatty acids and metabolic cofactors. These substances must pass through the chloroplast envelope in order to be utilised. It, therefore follows that the permeability properties of this membrane are of great importance. This has been investigated in relation to chloroplast terpenoids and its possible importance in gibberellin biosynthesis has been emphasised (Stoddart, 1968).

15. Agents of Control

The most dramatic changes in the plastid are induced by light. In addition to its effect on protochlorophyllide transformation it has been found to have short and long-term effects on chloroplast metabolism. After a brief period of illumination protochlorophyllide resynthesis shows a 5 - 10 minute lag period and then continues to the same level as before (Augustinussen and Madsen, 1965). It has been suggested that this may be the result of the temporary removal of feedback inhibition of δ-ALA synthetase activity (Granick, 1967). Pre-treatment with light (particularly red) followed by a dark period overcomes the longer lag period of chlorophyll synthesis (See Section 5 of Introduction). This lag period is also present in protochlorophyllide synthesis (Shibata, 1957; Butler, 1961; Augustinussen and Madsen, 1965) and is shortened by pre-irradiation (Virgin, 1958), suggesting that the effect on the lag period of chlorophyll synthesis is via protochlorophyllide synthesis. It has been indicated that red light may operate via ALA supply (Kirk and Tilney-Bassett, 1967) possibly through increased synthesis of RNA and enzymes of the δ-ALA synthetic pathway (Gassman and Bogorad, 1967). Red light treatment also promotes the formation of other chloroplast constituents such as plastid nitrogen, carotenoids and lipids (Mego and Jagendorf, 1961). Several chloroplast enzymes are induced by treatment with a brief duration of red light (Filner and Klein, 1968) and Bradbeer (1971) concluded that the increases in *P. vulgaris* were the result of *de novo* synthesis of proteins.

The primary site of action of red light is not known, however,

until recent years it has been thought that it acted at a nucleic acid level possibly by induction or de-repression of an operon. Fast responses to red light have been observed in systems involving permeability changes and this has been suggested as an alternative mechanism (Hendricks and Borthwick, 1967; Smith, 1970). The importance of the chloroplast envelope in plastid development has already been emphasized and this could be a suitable target for red light to activate.

Within the plant there exist modifying influences which alter the rate and level of chlorophyll synthesis. The presence of cotyledons enhances chlorophyll production in primary leaves of *P. vulgaris* (Wolff and Price, 1960; Sisler and Klein, 1963) and in cucumber (Hardy, Castelfranco and Rebeiz, 1970) and mustard (Moore and Lovell, 1970) cotyledons, chlorophyll production is controlled by the presence of the embryonic axis. The importance of the cotyledons in the photosynthetic development of bean chloroplasts was observed by Briggs (1920).

These reports suggest the presence of diffusible factors which are transported from the effective plant part to the greening organ. These factors may be induced (De Greef and Caubergs, 1972) or inhibited (Hardy *et al*, 1970) by light. Replacement of the plant organs with hormones such as IAA, GA_3 and 6-BAP was not successful (Sisler and Klein, 1963; Hardy *et al*, 1970) and cotyledon extracts were ineffective (Sisler and Klein, 1963). These were mainly preliminary investigations and do not preclude the possibility of a role. The literature suggests that GA_3 may increase (Wheeler and

Humphries, 1963; Negbi and Ruskin, 1966) and decrease (Sestak and Ullman, 1960; Artomonov, 1966; Szalai, 1968, 1969; Grebenskii and Palanitsa, 1970) chlorophyll synthesis in green and greening plant material. There are also reports which show that GA_3 enhances the retention of chlorophyll in senescing tissue (Lewis, Coggins and Garber, 1964; Fletcher and Osborne, 1966; Beevers, 1966; Whyte and Luckwill, 1966). The effects of exogenously applied cytokinins appear more consistent in their nature. In most cases kinins have stimulated the chlorophyll level (Sugiura, 1963; Banerji and Laloraya, 1971; Shlyk and Averina, 1969; Beevers, Loveys, Pearson and Wareing, 1970; Fletcher and McCullagh, 1971; Kaul and Sabkarwal, 1971; Penner and Wiley, 1972; Stobart, Shewry and Thomas, 1972), however, Narain and Laloraya (1970) observed a cytokinin - inhibited chlorophyll level. In senescing leaf material cytokinins are very effective agents in retarding the breakdown of chlorophyll (Richmond and Lang, 1957; Osborne and McCalla, 1961).

There is a clear indication that chlorophyll synthesis and chloroplast development are modified by diffusible internal factors and that known growth hormones can affect the chlorophyll levels in plants. The purpose of this thesis is to investigate the importance of growth regulators to primary leaf chlorophyll synthesis in *Phaseolus aureus* (mung bean) with reference to the role of other plant organs.

EXPERIMENTAL

EXPERIMENTAL

I. Materials

Mung beans were grown at 25°C in total darkness in vermiculite and prepared for experimentation at the appropriate stage. When only the primary leaves were required, they were detached from the plant immediately beneath the base of the petioles, thus including the apical bud in the system.

Experimental material incorporating the cotyledons was removed from the plant at the base of the cotyledons, while in hypocotyl treatments the roots and cotyledons were removed from the plants. The desired age of the material was determined from the growth curve shown in Figure 7 (Results Section).

Prior to use, the samples of leaves were matched by weight for size and age. With cotyledons and hypocotyls attached, this was not possible since the variation in the relationship between leaf weight and cotyledon or hypocotyl weight was large. In these cases the samples were matched subjectively. Unless stated otherwise, the samples consisted of five leaf pairs. These were placed on two layers of Whatman No.3 filter paper in 9 cm crystallizing dishes containing 10 mls of the appropriate treatment. After 4 hours dark incubation, the leaf pairs were illuminated under fluorescent tubes (1000 lux) at 25°C for the desired period of time.

An agar base was tried as a possible medium for incubation of the leaves. The latter were pushed into the agar by means of their short stems. This proved impracticable, mainly because the stems were not sufficiently strong to break the agar surface and support the leaf.

The gibberellins, GA_3, GA_4 and GA_7, were all dissolved in distilled water and used from a stock solution of 100 mg/l (2.9×10^{-4}M GA_3). Kinetin and 6-BAP were initially dissolved in DMF and made up to volume with distilled water. The final concentration of the cytokinins was 100 mg/l (4.4×10^{-4}M 6-BAP) containing 0.5% DMF. The highest concentration of DMF used in experiments did not affect chlorophyll synthesis. The growth retardants CCC and B9 (CIBA) were also made up in stock solution at 100 mg/l (2.5×10^{-4}M CCC and B9) in distilled water, but B9 (Uniroyal) was initially dissolved in methanol to give a final concentration of 2% in 100 mg/l solution. Carbohydrates were used from stock solutions at a concentration of 0.4M. CMU was made up to a final concentration of 10^{-3} M in 2% methanol. All solutions were stored at 4^{o}C and replaced at monthly intervals.

2. Fresh Weight

This was measured immediately after excess liquid had been blotted from the material.

3. Dry Weight

Fresh samples were dried for 24 hours at 100°C before being weighed. In experiments where chlorophyll was extracted from leaf material dry weight measurements were made on the extracted leaves. This resulted in decreased values for the dry weights, but was adequate for comparison between treatments.

4. Chlorophyll

This was extracted by soaking the leaves in 80% acetone and storing the samples at 1 - 2°C. The concentration of chlorophyll was determined spectrophotometrically using the method of Arnon (1949).

5. Cell Counts

Samples of five leaf pairs were macerated in 10 mls of 5% chromic acid (Dale, 1964) incubated for 20 hours at 40°C and the separated cells were counted in a haemocytometer. The proporation of chloroplast-containing cells in the leaves was obtained by counting the chlorophyllous and non-chlorophyllous cells of sections taken from distal and proximal regions of the leaf. Two regions of the lamina were analysed for each of the two sections per treatment. The sections were obtained using a freeze microtome.

6. Chloroplast Counts

Five pairs of leaves were fixed in 0.5% osmium tetroxide for 15 minutes and subsequently transferred to 10 mls of pectinase (2.5% Rohament P in 0.2M phosphate buffer at pH 5.6) at 38°C. Single cells were obtained after 4 hours incubation, at which time the chloroplasts were counted under phase contrast. Counting was facilitated if the coverslip was pressed gently to rupture the cell membrane (Possingham and Saurer,1969), thus compressing the chloroplasts into a single plane.

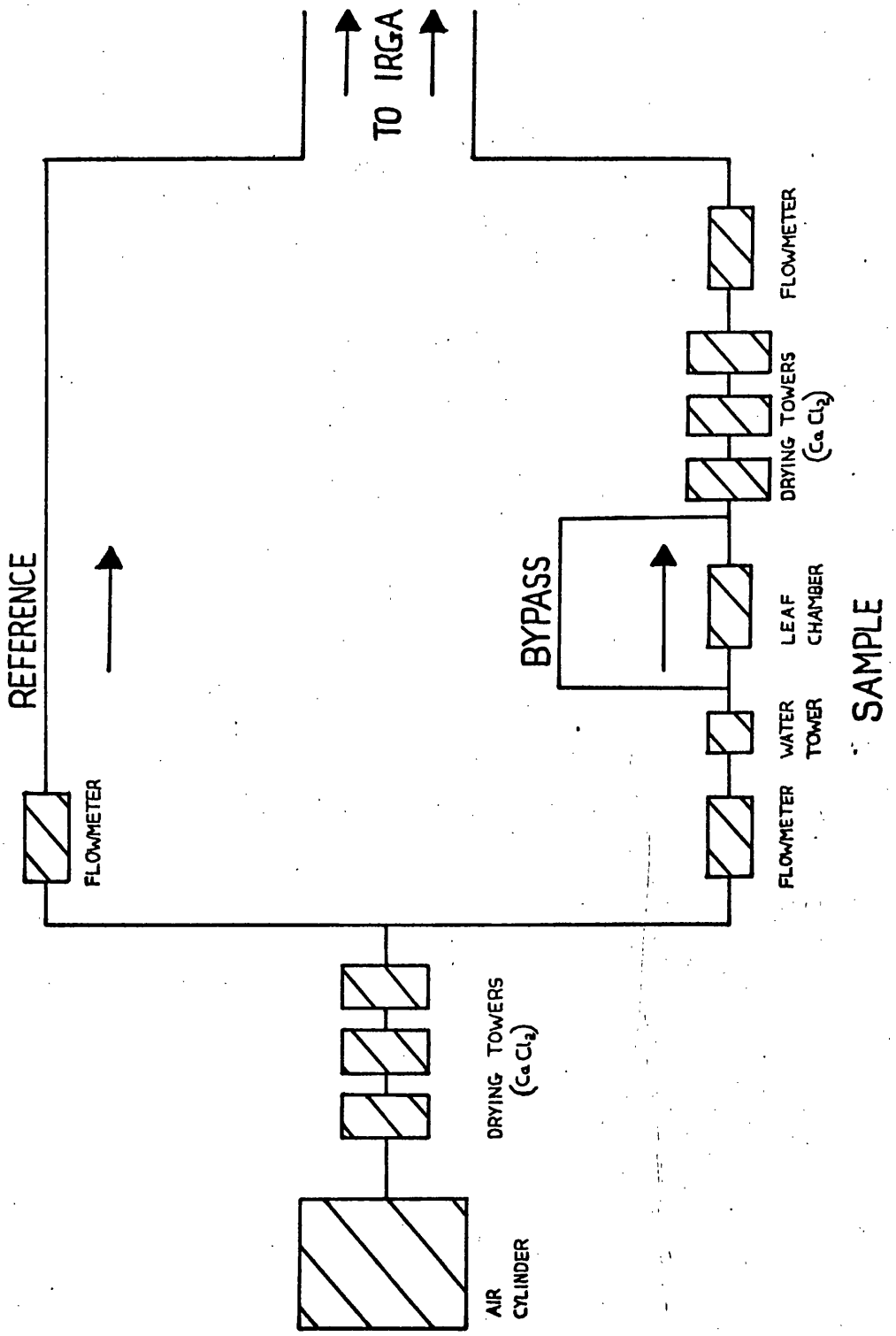
Although the chloroplasts were clearly visible, fixation was necessary to prevent their rupture when pressure was applied to the coverslip. Osmium tetroxide (0.5%) was found to be better than glutaraldehyde (3.5%) in this repect and both fixatives hindered cell separation necessitating a higher concentration of the pectinase enzyme. Hydrochloric acid (1 N) at 60°C (Possingham and Saurer,1969) was not very effective as a macerating agent for mung bean leaves.

7. Carbon Dioxide Analysis

The carbon dioxide exchange of leaf samples was measured using a Grubb Parson infra-red gas analyser (IRGA) linked to an open circuit gas flow system (see Diagram in Figure 5). Air from a cylinder was passed through a series of drying towers containing calcium chloride and then split into two separate lines, one, the 'reference' which led directly to the IRGA and the other,

FIGURE 5

DIAGRAM OF GAS FLOW FOR IRGA ANALYSIS



the 'sample', which fed the chamber in which the leaves were placed. The air stream was wetted prior to the chamber to prevent desiccation of the leaves and dried by calcium chloride towers placed after the chambers before being fed to the IRGA. A sample bypass was arranged around the chamber so that a baseline could be obtained. The air flow was controlled by flowmeters (G.A. Platon) in each line. As a precaution against leaks in the chamber, flowmeters were placed before and after the chamber in the sample line.

Each sample consisted of twenty leaf pairs and gas exchange was measured at a light intensity of 12,000 lux and a flow rate of 500 mls/minute.

The rate of photosynthesis was calculated from the following equation:

$$\frac{x \; Cf}{M \times 10^6} = y$$

where y = rate of CO_2 uptake in mls/min.

x = difference between light and dark traces (in recorder divisions)

M = Recorder/IRGA magnification

C = Calibration of one IRGA division

10^6 converts from ppm.

To calculate the respiration rate, x must represent the difference between the dark trace and the baseline. The rates in mls/min were converted to μ moles/hour and expressed in terms of the whole leaf, fresh weight, dry weight and/or chlorophyll.

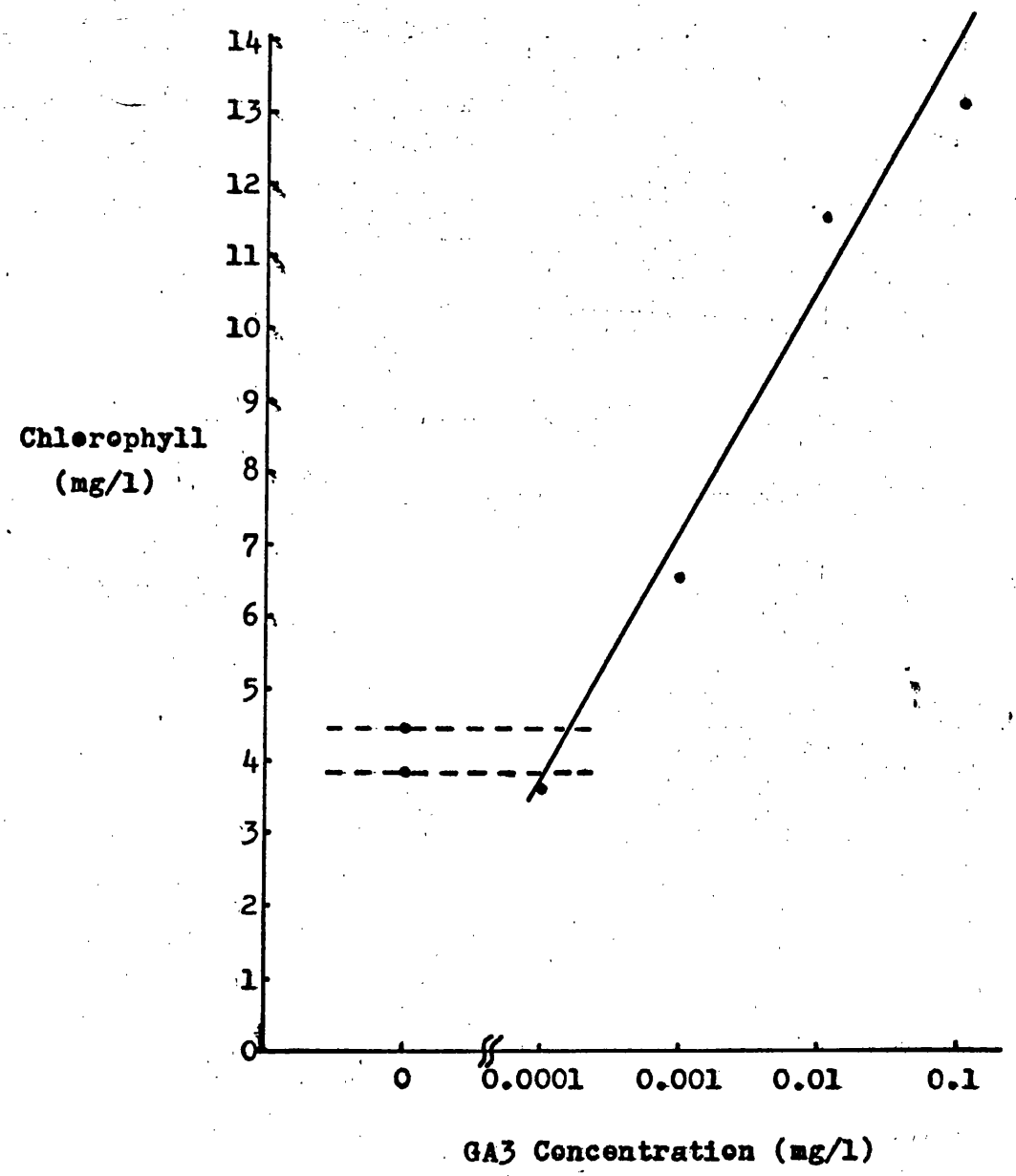
8. Endogenous Gibberellin Analysis

Four hundred mung bean leaves were extracted with 80% methanol and acid washed sand in a pestle and mortar. The debris was removed by centrifugation at 3,000 rpm for 15 minutes in a Griffin 'Christ'and the supernatant was evaporated under vacuum at 45°C until only the aqueous phase remained. The pH of this was adjusted to 2.5 with HCl and extracted four times with equal volumes of ethyl acetate. The ethyl acetate fractions were bulked and partitioned four times with 5% sodium bicarbonate. The latter fraction was collected and the pH adjusted to 2.5, then it was re-extracted four times with half volumes of ethyl acetate. This final extract was evaporated under vacuum to approximately 10 mls and then concentrated to less than 1 ml by evaporation under a stream of nitrogen. The samples were stored at -15°C until required for chromatography. At all stages during the extraction when it was necessary to temporarily store the extracts, this was done at 2 - 3°C.

The extracts were run on Whatman No.1 chromatography paper in isopropanol : ammonia : water (10 : 1 : 1). The chromatograms were cut into ten Rf strips, each of these was laid in a 5 cm Petri dish containing 1.5 mls of distilled water. Inert strips of chromatography paper were used in the control and GA_3 standards of 0.0001, 0.001, 0.01 and 0.1 mg/l were also set up. Gibberellin levels were measured using the Rumex leaf disc bioassay (Whyte and Luckwill, 1966). Discs were cut from eight Rumex leaves, using a No.4 cork borer, and one disc from each leaf was placed in each Petri dish. The dishes were then left in the dark for 5½ days, by which

FIGURE 6

CALIBRATION CURVE : RUMEX GIBBERELLIN BIOASSAY.
CHLOROPHYLL RETENTION OF GA3-TREATED LEAF DISCS



time the controls were sufficiently yellow. The chlorophyll from each batch of discs was extracted in 80% acetone and measured as stated previously. The calibration curve obtained from the standard GA_3 concentration is shown in Figure 6.

9. Expression of Results

The results were expressed as units of chlorophyll or CO_2 in the whole leaf and in relation to the fresh weight and dry matter content of the leaves. In experiments concerning the effect of the cotyledon and hypocotyl, fresh weight was used since both fresh and dry weight of the leaf increased. When leaves were incubated alone fresh weight increased and dry weight decreased. Hormones supplied to these leaves caused greater water uptake than occurred in the controls. To avoid expressing the chlorophyll content in terms of the extra water content of the leaves, the dry weight was used for calculations of concentration. Chlorophyll content per leaf was estimated to determine the effect of the treatments on overall chlorophyll level, while the concentration was considered to represent the degree of effect related to the remainder of leaf. The changes in chlorophyll elicited by the various treatments were expressed as a percentage of the appropriate control level. This was necessary because of the variation in control levels between experiments.

RESULTS

RESULTS

I. Growth of Plants

In this section results from the general growth parameters of dry weight and fresh weight are recorded in order to establish the inter-relationships of the various organs of *P. aureus* in the dark and the light.

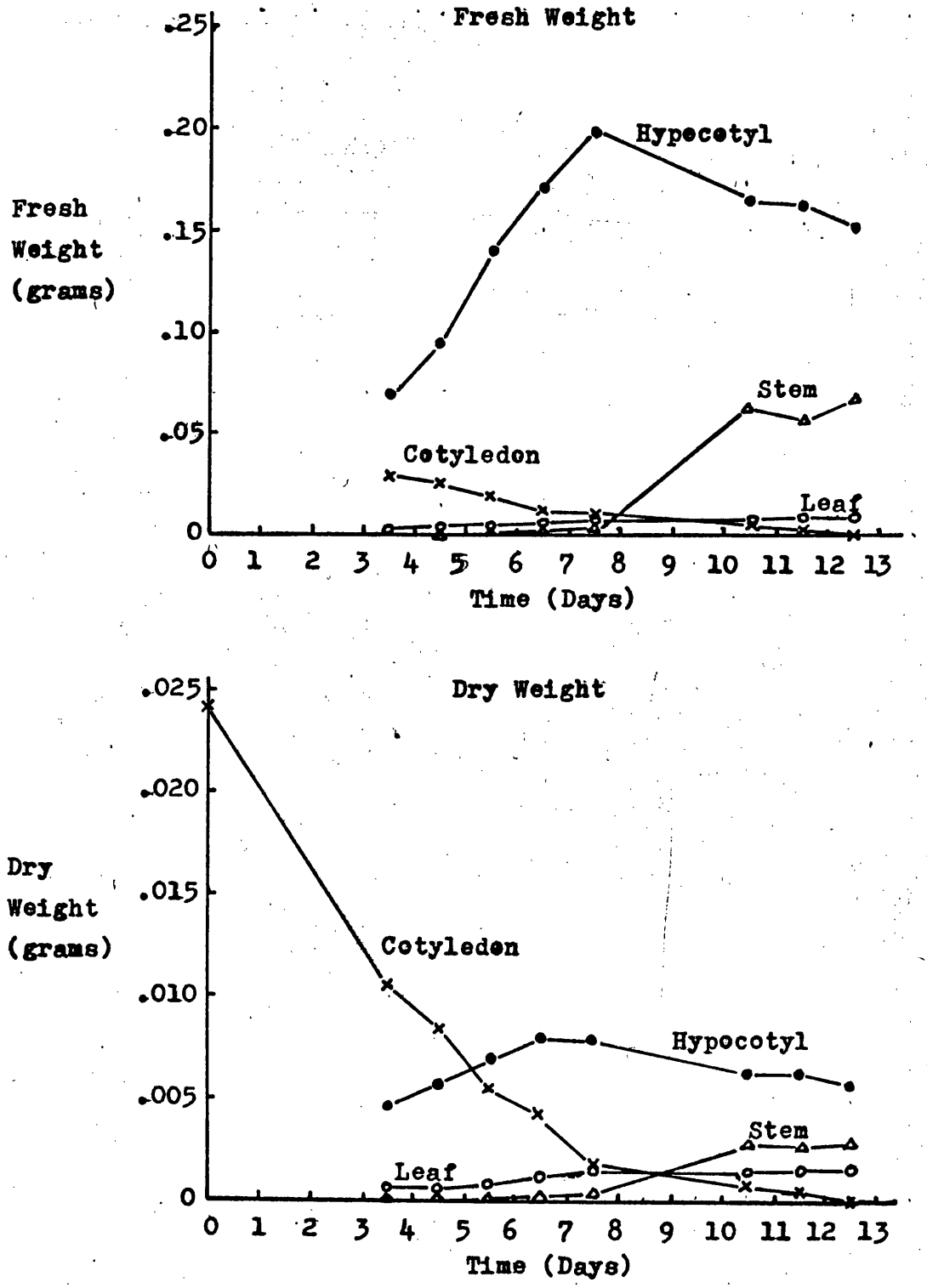
During etiolated growth, the hypocotyl, stem and leaf developed at the expense of the cotyledon (Figure 7) which was almost expended after 8 days and had disappeared after 12 days. The dry weight increase of the hypocotyl ceased after 6½ days, just prior to the end of the cotyledons useful life, and was followed by a decrease which corresponded to the phase of rapid increase in dry weight of the stem. Leaf development was very gradual for the first 6½ days and then reached a plateau. The fresh weight data have also been included to illustrate the very large water uptake and extension which occurred in the hypocotyl and stem relative to the leaf and cotyledon fresh weights.

From this circumstantial evidence it may be suggested that during etiolated growth the cotyledon directly supports the development of the hypocotyl and leaf while the stem develops from the reserves of the hypocotyl.

In subsequent experiments, the stated age of the material used, refers to the weight development as depicted in Figure 7.

FIGURE 7

GROWTH PATTERN OF ETIOLATED MUNG BEANS



The relationship of the cotyledons and leaves during illumination was investigated by detaching this system immediately beneath the point of attachment of the cotyledons to the hypocotyl, thus excluding the latter and the small root system. This system will be referred to as the cotyledonary explant. The effect of detachment (after 6 days growth) on etiolated growth of the explant during the next 48 hours (Figure 8) was to terminate the increase in leaf dry weight and to slow the loss of cotyledon dry weight. When incubated alone (Figure 9), the leaves showed a loss in dry weight and the cotyledons a further retardation of weight loss, thus demonstrating that the cotyledons were just maintaining the leaves after detachment. Some of the loss of cotyledon weight was probably due to the presence of the stem, the weight of which was not recorded.

The effect of illumination at 1000 lux for 48 hours on the cotyledonary explant varied with age (Figure 8). Four-and-a-half day old leaves increased very rapidly in dry weight until after 48 hours they were twice as heavy as their counterparts on the etiolated plant. Five-and-a-half day old leaves increased in dry weight at a similar rate to those on the whole plant in the dark, but continued their increase for 48 hours and eventually rose above the plateau level for those etiolated leaves. They were about 70% heavier than the leaves of the cotyledonary explant incubated in the dark. After 6 days of etiolated growth, the leaves of the illuminated explant did not increase in dry weight when compared with the etiolated leaves of the whole plant and they decreased in weight when eight-and-a-half day old cotyledonary explants were illuminated. Leaves from all ages, when incubated alone in the light (Figure 9), decreased in dry weight

FIGURE 8

DRY WEIGHT CHANGES IN LEAF AND COTYLEDON
UNDER VARIOUS CONDITIONS

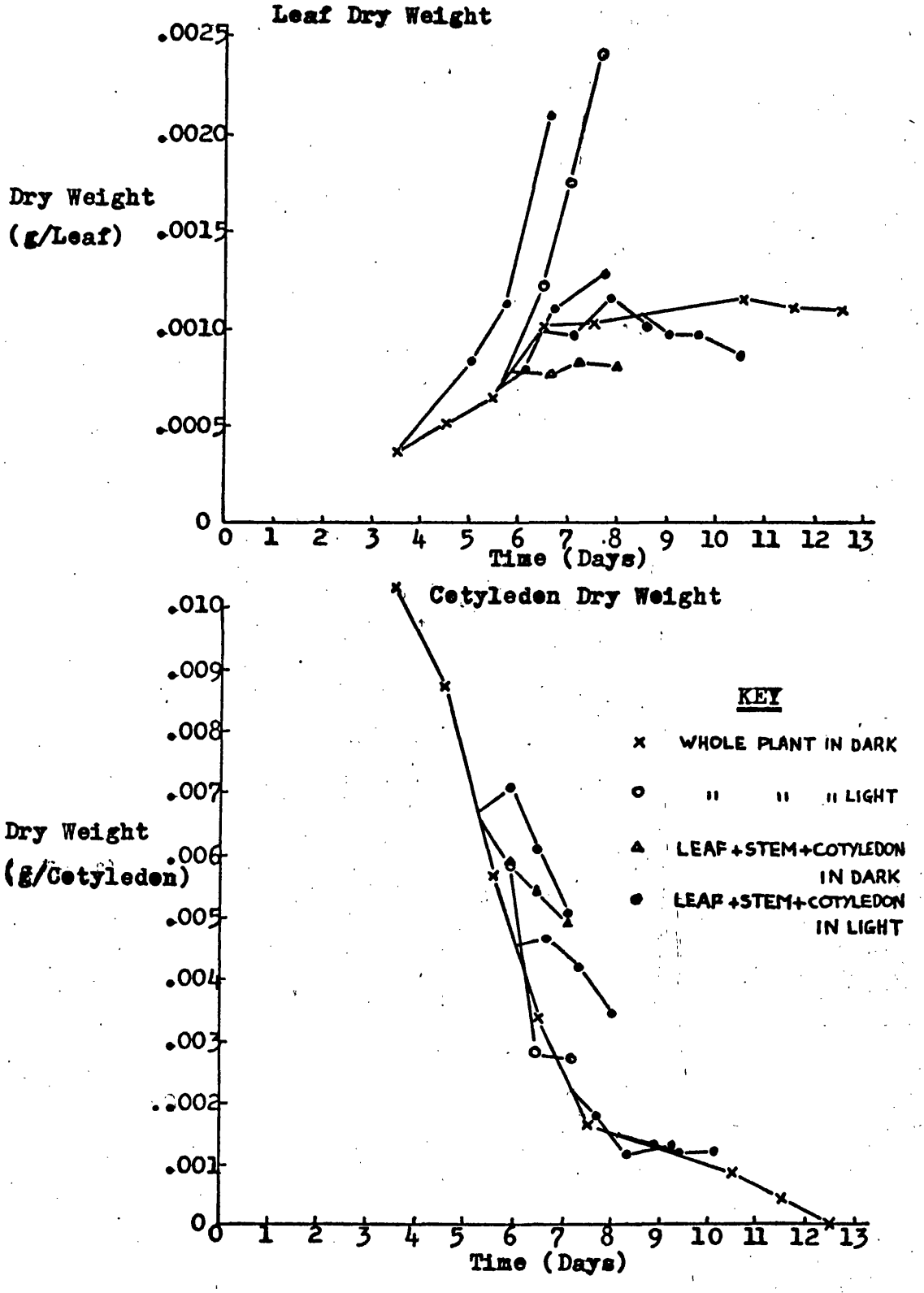
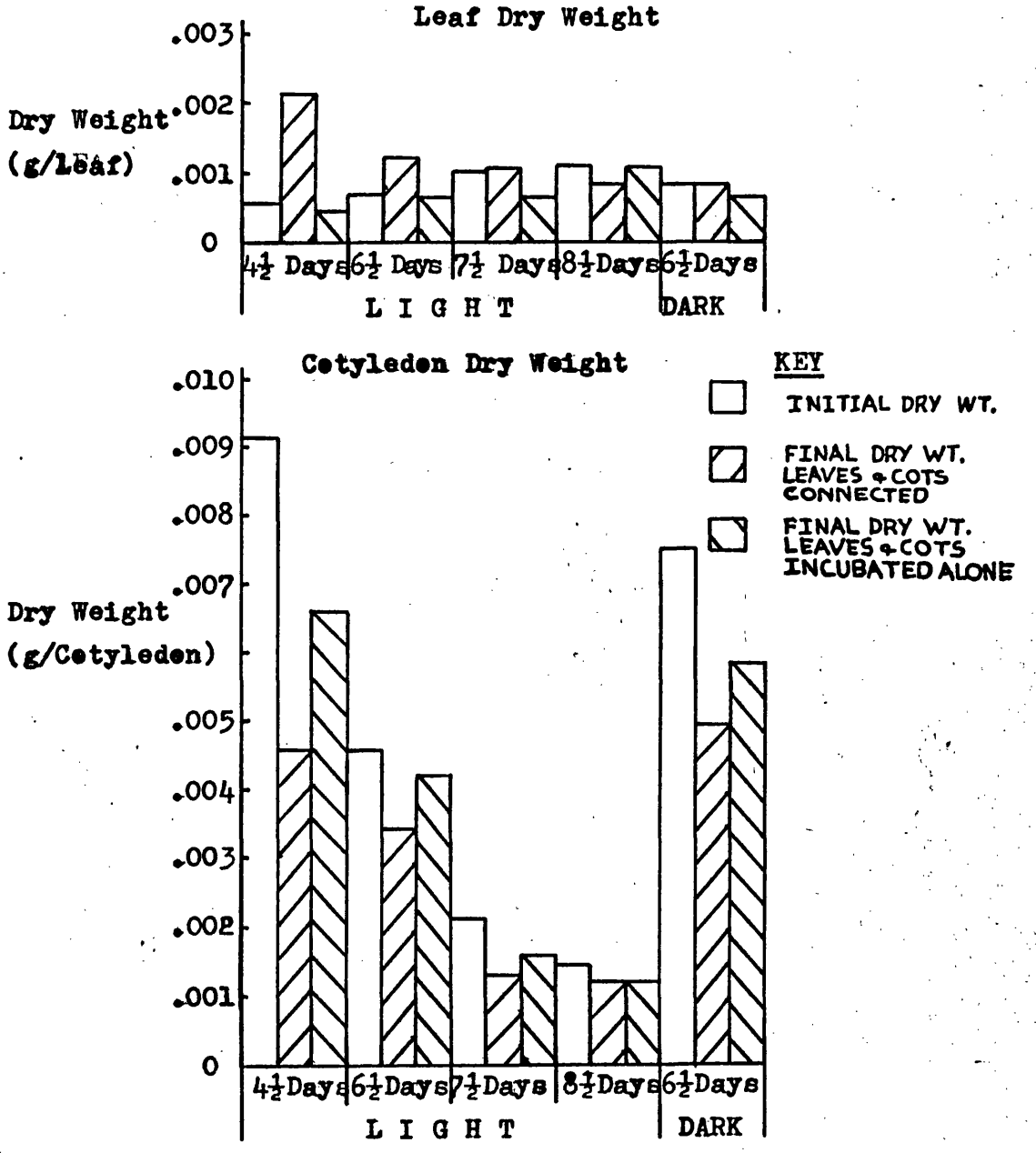


FIGURE 9

DRY WEIGHT CHANGES IN LEAF AND COTYLEDON
AFTER 48 HOURS IN LIGHT(1000 LUX) OR DARK



The cotyledon+stem+leaf system was removed at the stated age of dark growth and incubated either as the complete system or in separation.

relative to their initial value. Leaves from the three younger ages confirmed that the presence of an effective cotyledon was essential for weight increase to occur. This is compatible with the results of Wheeler (1966) who found that the cotyledons affected expansion of the primary leaves of *P. vulgaris* only if they were removed prior to five days of light growth. In the oldest mung bean leaves, the decrease in leaf dry weight was greater in the presence than in the absence of the cotyledons, suggesting that at this stage the cotyledons were hindering leaf development. A comparison of the dry weight of dark- and light-incubated 6 day old leaves shows that light altered leaf dry weight only in the presence of the cotyledons. The cotyledons must, therefore supply the leaves with factors necessary for their response to light.

These changes were reflected in the dry weight changes of the cotyledon. On illumination of the explant the cotyledons lost weight at all ages. The $7\frac{1}{2}$ and $8\frac{1}{2}$ day old cotyledons lost weight in a similar manner to the cotyledons of the etiolated leaf but the cotyledons from $3\frac{1}{2}$ and $4\frac{1}{2}$ day old illuminated explants exhibited the same retardation of weight loss observed in the dark-incubated explant. Since leaf development in the former is so much larger than in the latter, light evidently caused the supply of cotyledonary reserve to be utilized more efficiently by the leaf. A feature of the time-course for the loss of cotyledonary dry weight at these ages is the lag observed during the first 16 hours. A small lag in the dry weight development of the leaves at these ages is also discernible. Since this lag was not present in the dry weight curve for the cotyledons of the dark-incubated explants it may be concluded

that it was light induced. The data in Figure 9 confirm that the presence of the leaf was the cause of the increased dry weight loss of the cotyledon. Cotyledons incubated alone lost less dry weight than those on the explants, with the exception of those which were eight-and-a-half days old, when the two weights were equal.

For comparison, five-and-a-half day old whole plants were illuminated for 48 hours and the dry weight changes in the leaves and cotyledons recorded (Figure 8). Leaf dry weight increased very rapidly without any lag phase and the final dry weight was greater than that for the leaves of four-and-a-half day old explants. The loss in cotyledon dry weight also exhibited no lag phase and decreased in a similar manner to that of the cotyledons of the etiolated whole plant. These results indicate that the hypocotyl also contribute substantially to leaf development in the light and may have had a large effect on the leaves at later stages as well. The absence of an obvious lag phase in the loss of cotyledon dry weight suggests that the lag present in the illuminated cotyledon explant was a feature of illumination in the absence of the hypocotyl.

The loss of effect of the cotyledon after 6 days etiolated growth occurred at a similar time to that for the cotyledons of *P. vulgaris* (Vyvyan, 1924; Wheeler, 1966) and *Capsicum annum* L. (Rylski and Halevy, 1972) during illuminated growth. The cotyledons of light-grown French beans persisted for eleven to sixteen days, (Öpik and Simon, 1963). This compares well with the 12 days which elapsed before the cotyledons of dark-grown mung beans finally disappeared. Although there is a difference in species

these comparisons and the results for loss of cotyledon dry weight in Figure 8 suggest that illumination does not affect the decrease in cotyledon dry weight on the whole plant.

It may be concluded that light stimulated the flow of substrate from the hypocotyl to the leaf while the cotyledons continued to supply the hypocotyl as they had in the dark. After detachment the cotyledons were able to promote leaf development in the light but this required an adjustment of the direction of flow of substrate and resulted in the observed lag phase. In Figure 10 the results of an investigation of these changes, including hypocotyl dry weight, are shown for 5 day old plants. The leaf dry weight changes show that the presence of the cotyledon and hypocotyl together was more effective than the two alone, and that their effect was additive. The hypothesis of substrate flow previously suggested was supported by the hypocotyl dry weight changes. In the presence of the cotyledon, the dry weight of the hypocotyl remained fairly constant, but decreased considerably in its absence. The data for cotyledon dry weight agree with that shown in Figure 8, i.e. the presence of the hypocotyl considerably enhanced cotyledon dry weight loss.

The older leaves showed little response in their dry weight changes when the cotyledonary explants were illuminated. It may be assumed that this was due to the loss of cotyledon reserve, however there exists the possibility that the response of the leaf had changed. For this reason 8 day old leaves were incubated on 0.2 M sucrose and illuminated for 48 hours (Figure 11). Six day old leaves were also examined for comparison. The graphs reveal that

FIGURE 10

DRY WEIGHT CHANGES OF LEAF, COTYLEDON AND HYPOCOTYL DURING 48 HOURS ILLUMINATION AT 1000 LUX

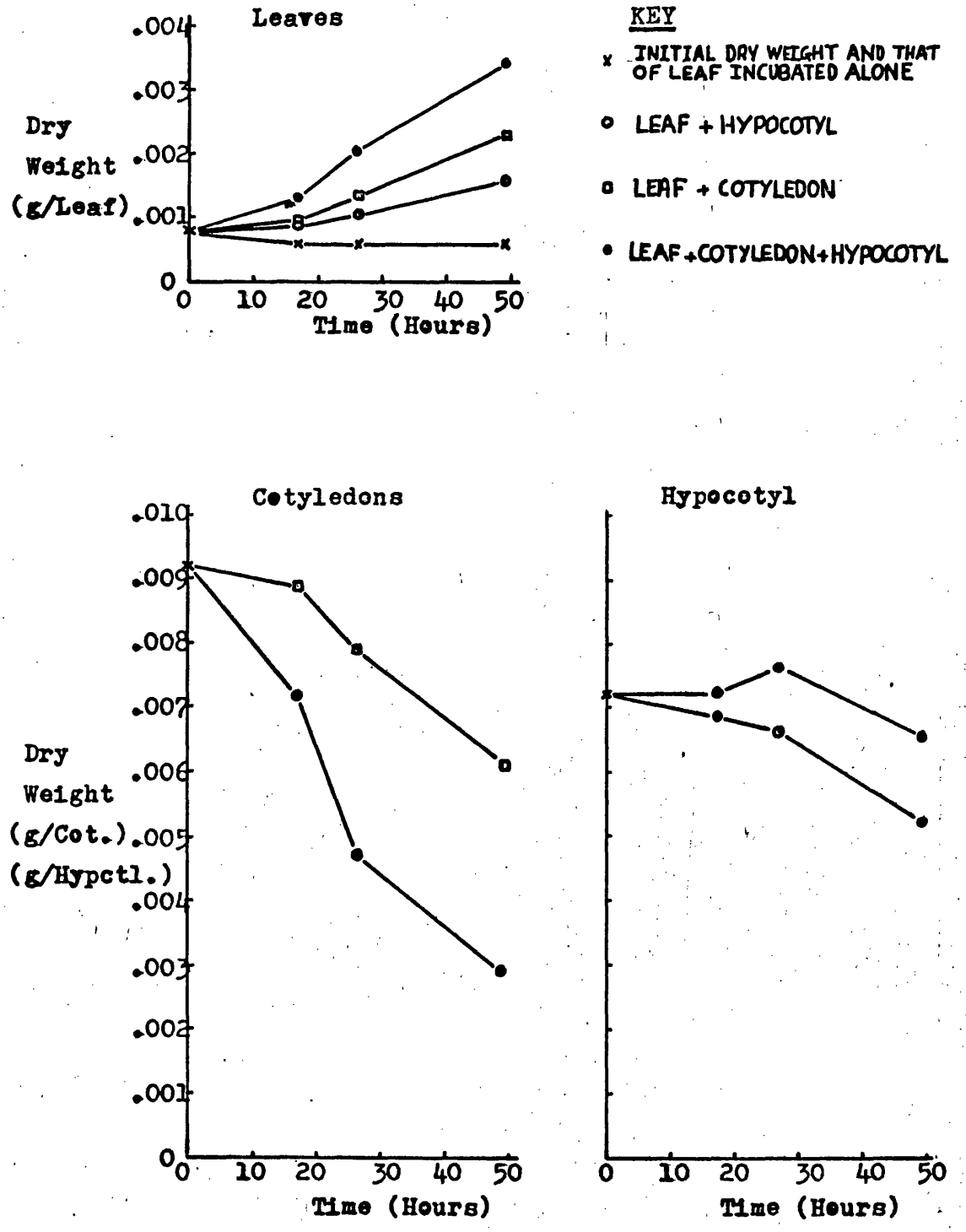
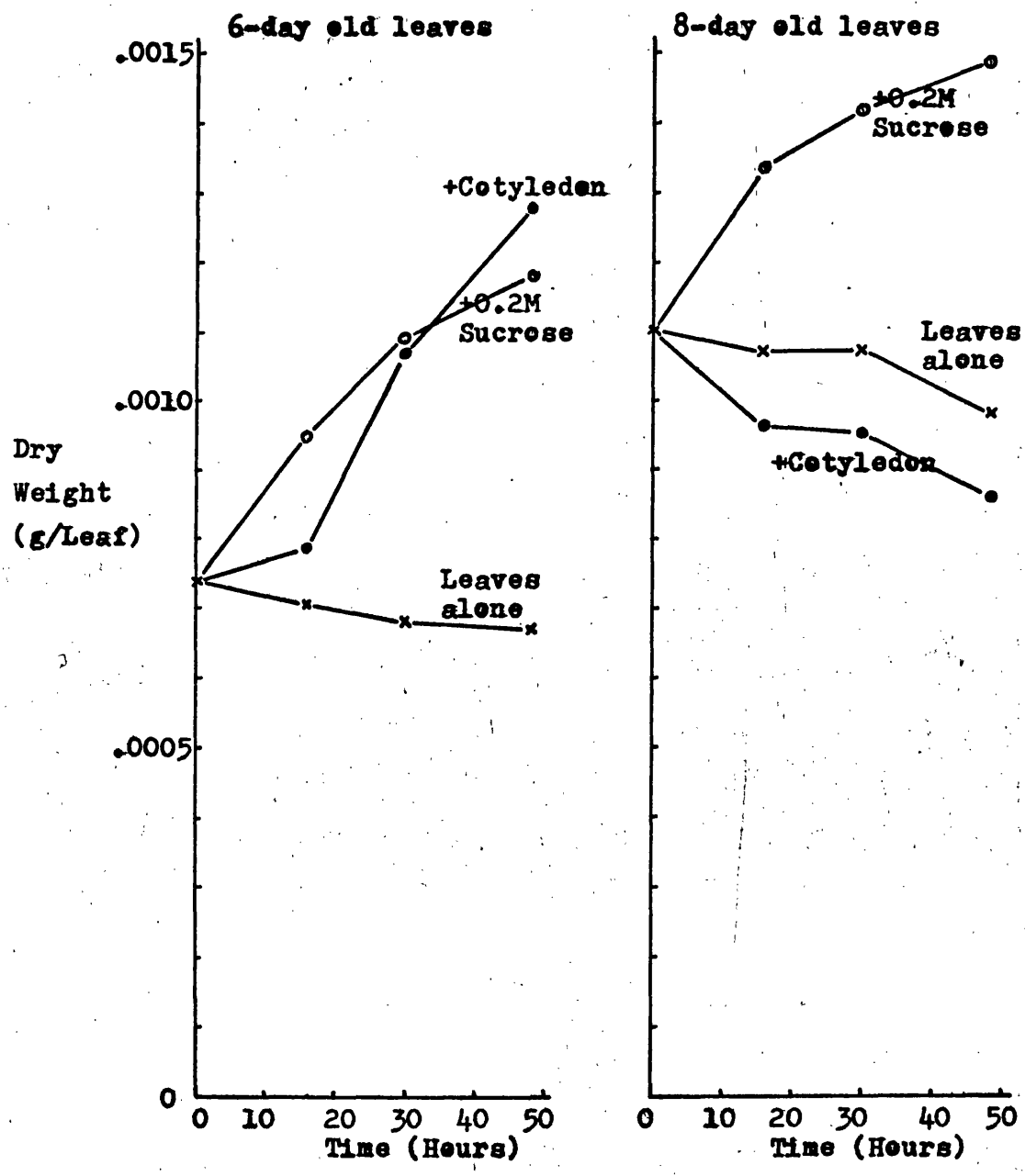


FIGURE 11

EFFECT OF 0.2M SUCROSE ON LEAF DRY WEIGHT
DURING 48 HOURS ILLUMINATION AT 1000 LUX



leaves of both ages responded similarly and both showed a substantial increase in dry weight. Both curves showed a rapid initial rate which gradually declined when compared with the shape of the curve for the leaves of the cotyledonary explant which exhibited a lag phase followed by a rapid increase. These results illustrate two points: firstly that the 8 day old leaves are capable of responding to substrate and that sucrose does not control leaf development in the same way as the cotyledons.

In Results Section 2(a) the experiments investigating the role of the cotyledons have been repeated to measure their effect on chlorophyll synthesis and these are compared with the effect of sucrose at the same ages.

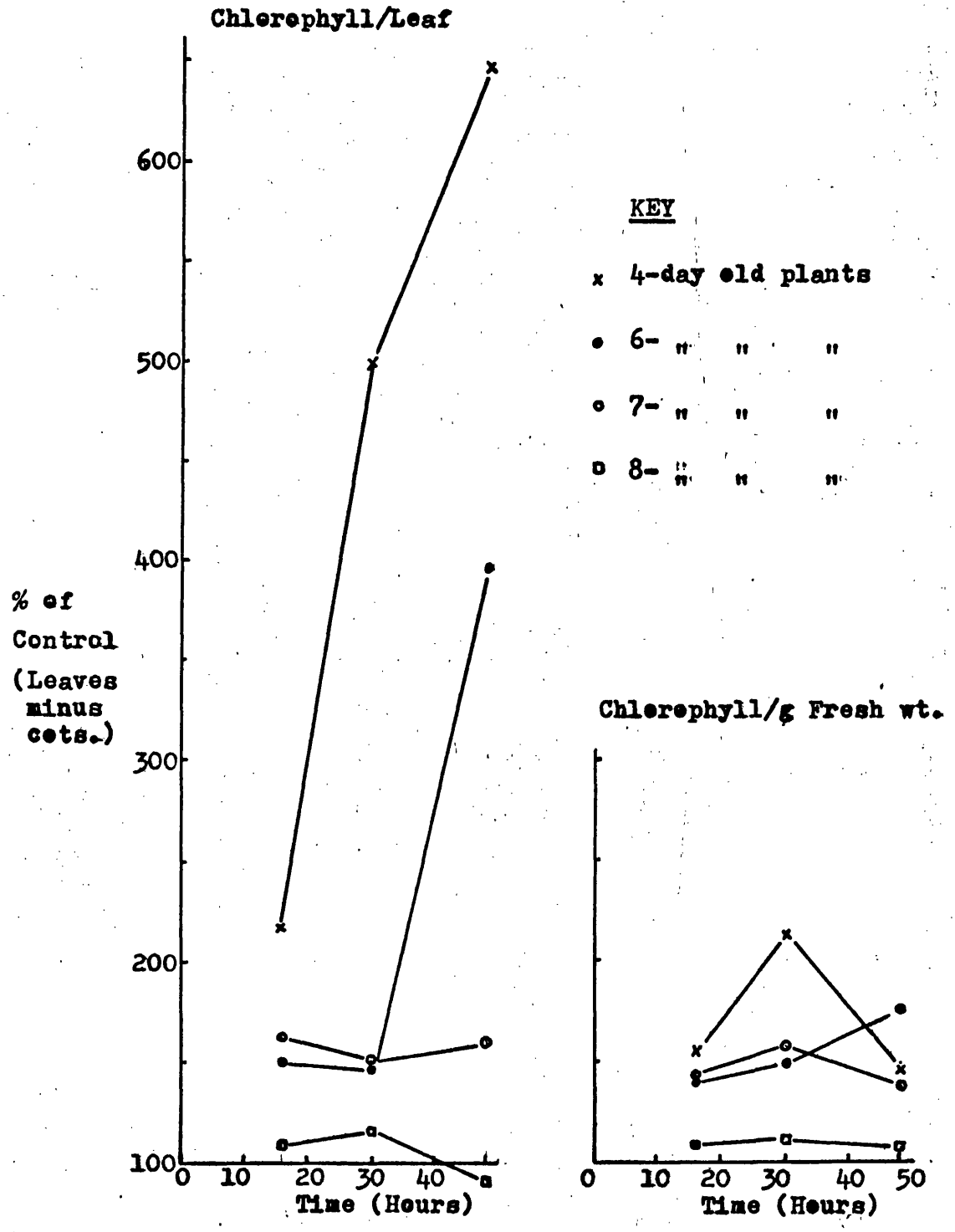
2. Chlorophyll Synthesis in the Primary Leaves

a) Effect of Cotyledon and Hypocotyl

The presence of the cotyledon enhanced chlorophyll synthesis at all ages examined, whether chlorophyll was expressed on a per leaf or per gram fresh weight basis (Figure 12). In 4 day old leaves illuminated with attached cotyledons the total chlorophyll content per leaf increased throughout the 48 hours incubation period. After a short lag the effectiveness of the cotyledon at six days also increased but was less than half that of the four day old cotyledons. In the seven day old leaves, the effect was fairly constant but much smaller than at 6 days. After 8 days growth the

FIGURE 12

EFFECT OF COTYLEDONS ON CHLOROPHYLL SYNTHESIS
IN PRIMARY LEAVES



effect of the cotyledon was very small and became inhibitory after 48 hours illumination. The size of the effect in the youngest plants was a result of the enormous leaf expansion induced by the cotyledon. On a fresh weight basis its effect reached 210% of the control after 30 hours incubation but then decreased to 150% after 48 hours. This was in great contrast to the total per leaf. The six day old leaves also showed a less dramatic rise when expressed on a fresh weight basis. The effect, however, did increase throughout the incubation period. Seven day old cotyledons also proved to be fairly effective on this basis, maintaining a 50% increase throughout. As before the 8 day old cotyledons elicited only a small increase.

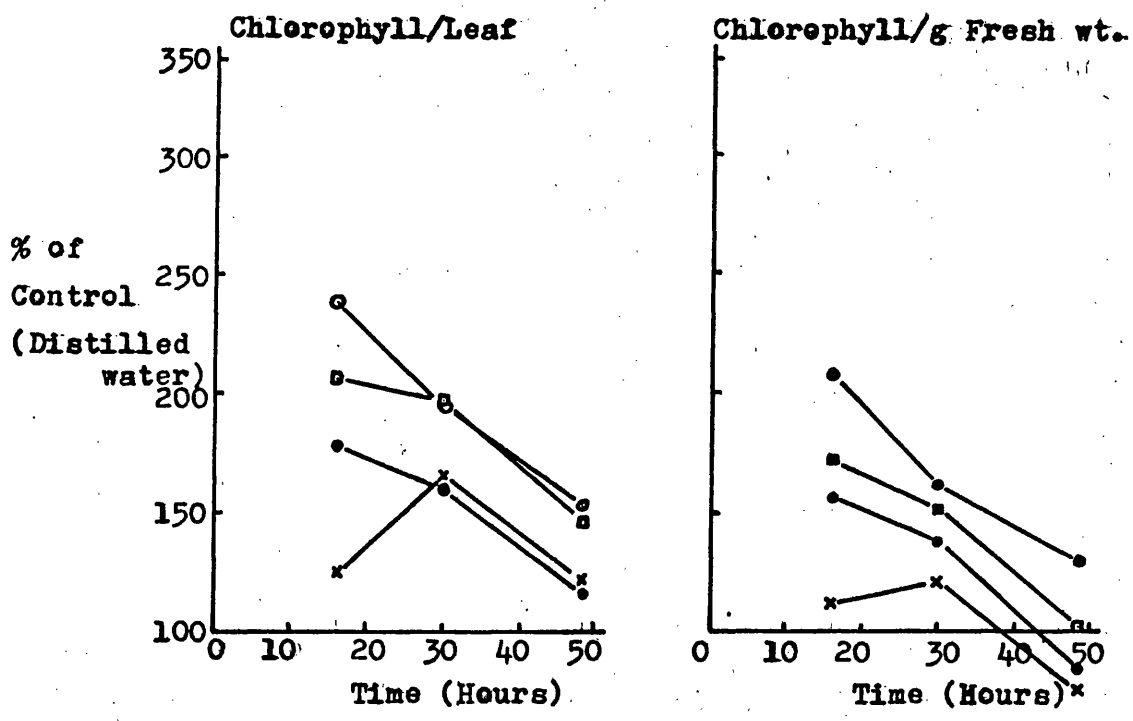
It may be concluded that the chlorophyll content of the whole leaves was decreasingly affected by the cotyledon as it aged, mainly because of the loss of effect on leaf expansion. On a fresh weight basis the effectiveness was not lost so rapidly and in fact, proved to be slightly greater, after 48 hours illumination, in 6 day old explants. It appears that during the life of the cotyledon either the balance of factors exported from the cotyledon or the response of the leaf altered in such a way that in the four day old explant leaf expansion was promoted to a greater extent than chlorophyll synthesis while in 6 day old explants the reverse occurred. This observation correlates with the conclusion of Sisler and Klein (1963) that a chlorophyll-promoting factor was exported from the cotyledon of *P. vulgaris* to the primary leaves at about the 5th day of etiolated growth.

The age effect of the cotyledons on chlorophyll synthesis in the primary leaves was similar to their effect on the dry weight development of the leaves. This suggests that any cotyledonary factors were general rather than specific in their effects. The percentage increases for dry weight were, however, less than those for chlorophyll per leaf.

The use of 0.2 M sucrose (Figure 13) as a replacement for the cotyledon revealed that the older leaves (seven and eight day) responded considerably more than the younger ages (four and six day). The shapes of the curves for chlorophyll per leaf indicated that in all but 4 day old leaves the maximum effect occurred during the early stages of illumination and gradually decreased. The curve for four day old leaves suggests that they developed a responsiveness to sucrose during the incubation period, since the maximum effectiveness occurred after 30 hours incubation. From this point it declined as at the other ages. These results could be due to either an increasing response to sucrose or a depletion of substrate with ageing. On a fresh weight basis, similar curves were obtained, although the percentage changes were somewhat reduced. After 48 hours incubation, 4 and 6 day old leaves showed inhibited chlorophyll levels on this basis. The effect of sucrose on chlorophyll synthesis would therefore appear to be immediate, and temporary. The difference in the shapes of the curves for the cotyledon and sucrose treatments and the different responses of the young leaves to these treatments suggests that sucrose alone was insufficient to replace the cotyledon in its effect on chlorophyll synthesis. This finding is similar to that for the effect on dry weight and agrees with the results of

FIGURE 13

EFFECT OF 0.2M SUCROSE ON CHLOROPHYLL SYNTHESIS IN PRIMARY LEAVES



KEY

× 4-day old plants

• 6- " " "

o 7- " " "

▫ 8- " " "

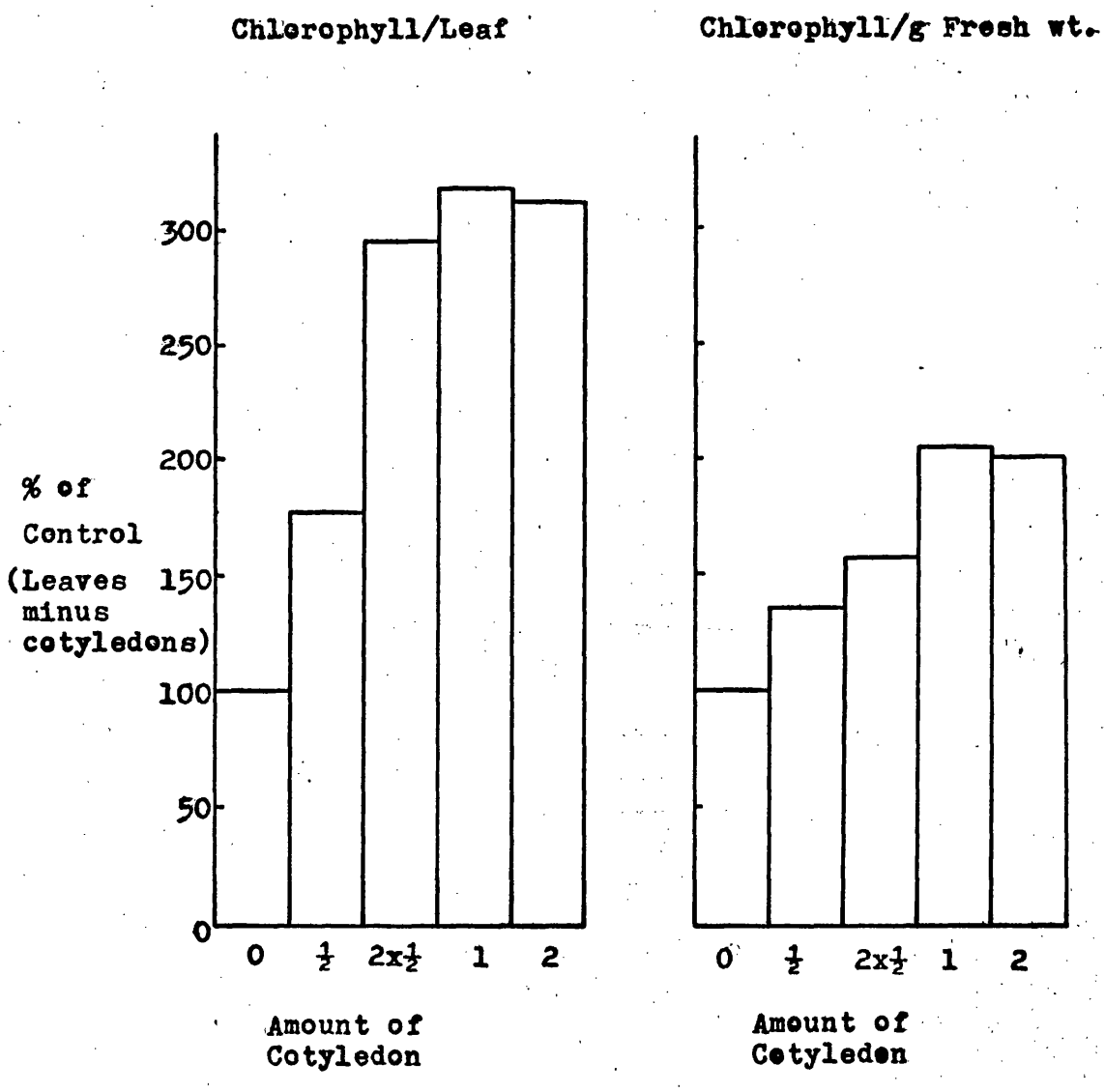
Wolff and Price (1960) and Sisler and Klein (1963).

Investigating the effect of ageing cotyledons on chlorophyll synthesis in primary leaves involved two variables; the decreasing supply of cotyledonary factors and a metabolically changing leaf. The experiments with sucrose showed that the dry weight of six and eight day old leaves was similar but that chlorophyll synthesis in the latter responded to a greater degree. To alter the cotyledon supply at a constant leaf age, five day old leaves with varying amounts of cotyledon were illuminated (Figure 14). Two half and two whole cotyledons elicited an increase which was slightly less than that elicited by one cotyledon. The presence of half of a cotyledon resulted in a promotion which was less than 50% of those due to the other treatments. The results on a fresh weight basis were essentially the same. The main conclusion is that the cotyledonary reserve at this stage was at a saturation level when only one cotyledon was present. Above this level no further increase was observed and below it the effect gradually decreased. There was no differential response of dry weight increase and chlorophyll synthesis to the different amounts of cotyledons. This contrasted with the differential effect observed with cotyledons of different ages and reinforces the suggestion that as the plants age the role of the cotyledon changes in relation to the leaf.

In Results Section 1 it was suggested that substrate from the cotyledons may first pass to the hypocotyl before being transported to the leaf rather than directly to the latter organ. It was thought that the chlorophyll level might reflect this relationship and so

FIGURE 14

EFFECT OF VARYING THE AMOUNT OF COTYLEDON ON CHLOROPHYLL CONTENT OF PRIMARY LEAVES



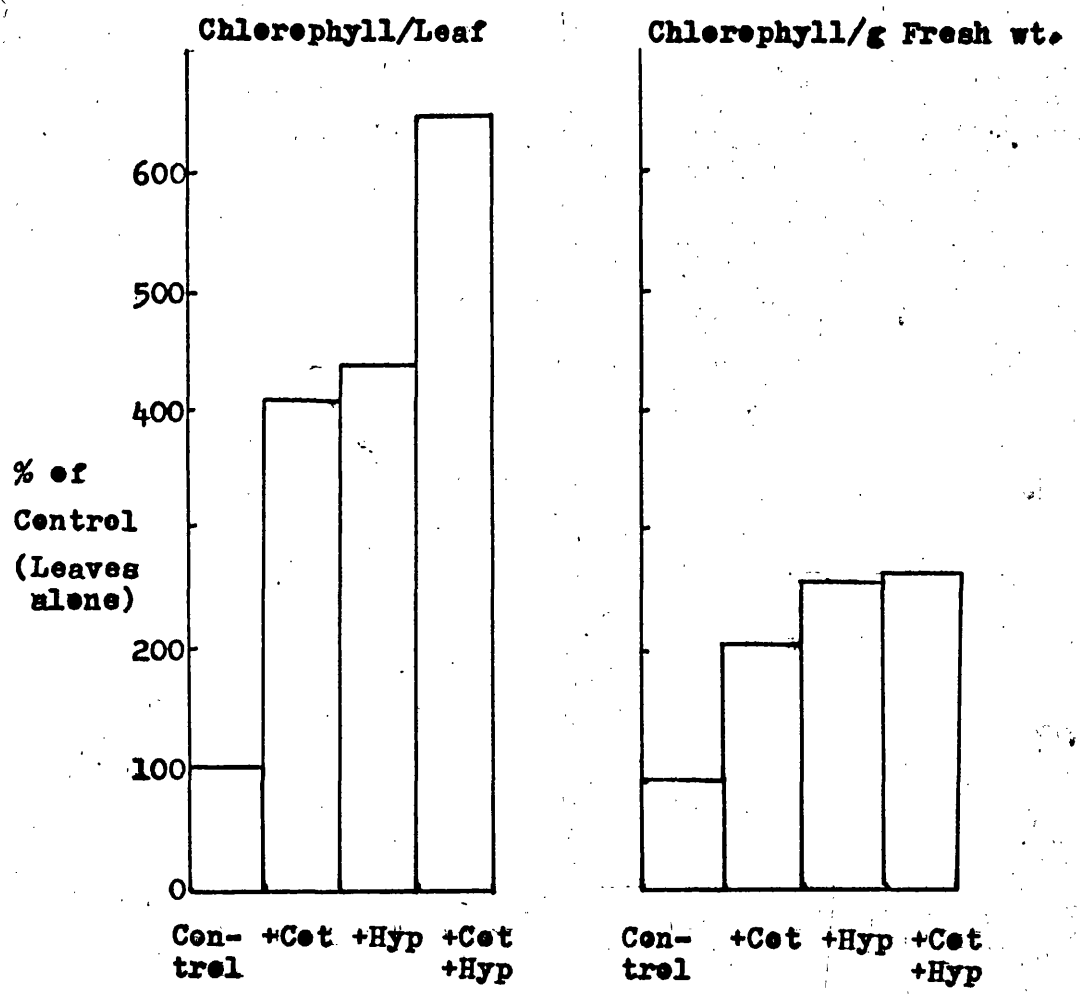
NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Explant from plants aged 5 days

the effect of the hypocotyl and cotyledon alone and together was ascertained (Figure 15). At five days, the hypocotyl was a more effective promoter of chlorophyll synthesis than the cotyledons, but the levels were of a similar order. Together, they further enhanced chlorophyll per leaf but the effect was less than additive, indicating some form of antagonism. On a fresh weight basis the hypocotyl was much more effective than the cotyledon and was almost as effective as the two together. These results suggest that at this stage of development the cotyledon was promoting leaf expansion with a resultant increase in chlorophyll synthesis while the hypocotyl more specifically promoted the latter. The effects at earlier and later stages would have provided interesting comparisons.

It may be concluded that while a large proportion of the effect due to the cotyledons and hypocotyl was the result of substrate supply, this was not their only role. The possibility of growth hormones forming part of the cotyledonary factor has been investigated for both leaf expansion (Wheeler, 1966) and chlorophyll synthesis (Sisler and Klein, 1963) in *P. vulgaris*. Gibberellic acid was implicated as a possible component of the 'leaf expansion' factor but neither gibberellic acid nor cytokinins affected chlorophyll synthesis. This was, however, examined only during the lag phase and at one age. Evidence from other sources indicates that both gibberellic acid and kinins may be effective in controlling chloroplast metabolism (see Introduction and Discussion). Bean cotyledons contain large quantities of gibberellins (Wheeler, 1960) and by inference, roots and cytokinins have a similar effect on leaf

FIGURE 15

INTERACTION OF COTYLEDONS AND HYPOCOTYL
IN AFFECTING
CHLOROPHYLL SYNTHESIS IN PRIMARY LEAVES



NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Plants aged 5 days

metabolism (Chibnall, 1954; Richmond and Lang, 1957; Kulaeva, 1962; Feierabend, 1969). It was therefore decided to investigate the effects of giberellins and cytokinins on leaf chlorophyll synthesis.

b) Effects of GA_3 and 6-BAP on Chlorophyll Synthesis

The role of the hormones was examined in four systems of differing substrate status:

a) in the presence of photosynthesis (control system)

b) in the absence of photosynthesis (plus CMU)

c) in the presence of exogenous substrate and photosynthesis (plus sucrose)

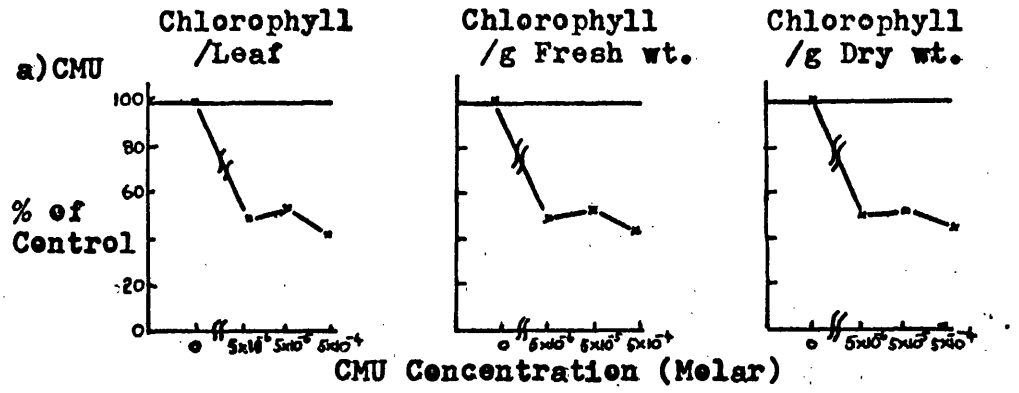
d) in the presence of exogenous substrate minus photosynthesis (plus sucrose/CMU).

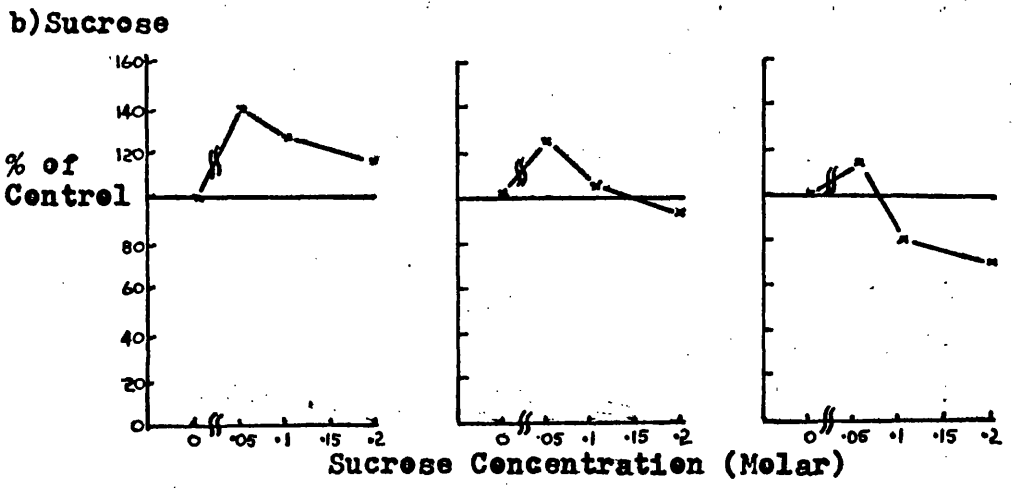
The competence of CMU to block photosynthetic reactions has been previously reported (Jagendorf and Margulies, 1960). It was hoped that the behaviour of the hormones in these systems might reveal some indication of the nature of their effects in addition to empirical data.

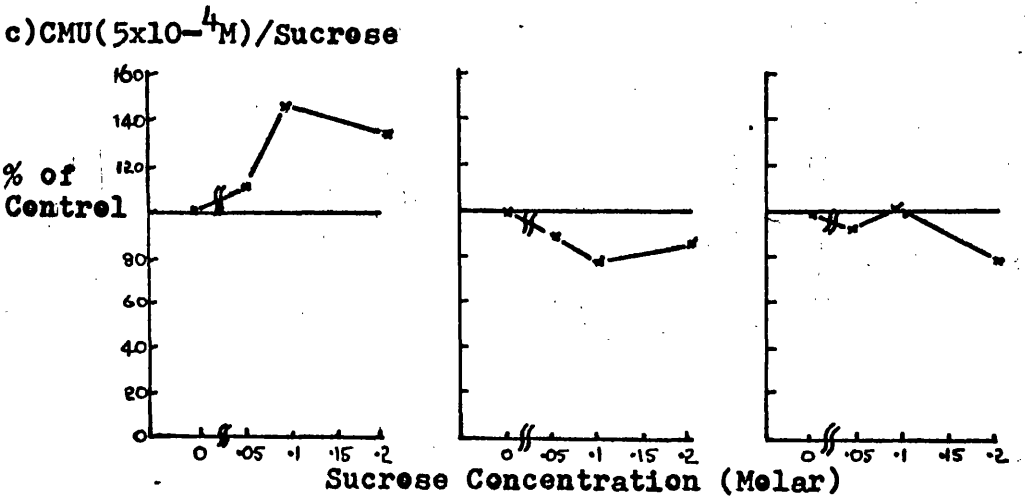
Figure 16 shows the effect of various concentrations of CMU and sucrose when applied to greening leaves. At the three concentrations of CMU tested (5×10^{-6}, 5×10^{-5}, 5×10^{-4}M) chlorophyll content was inhibited to approximately 50% of the control level. The greatest inhibition was at 5×10^{-4}M CMU and the pattern was similar for chlorophyll content plotted on each basis. The

FIGURE 16

EFFECT OF CMU, SUCROSE AND CMU/SUCROSE ON CHLOROPHYLL LEVEL OF PRIMARY LEAVES







NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

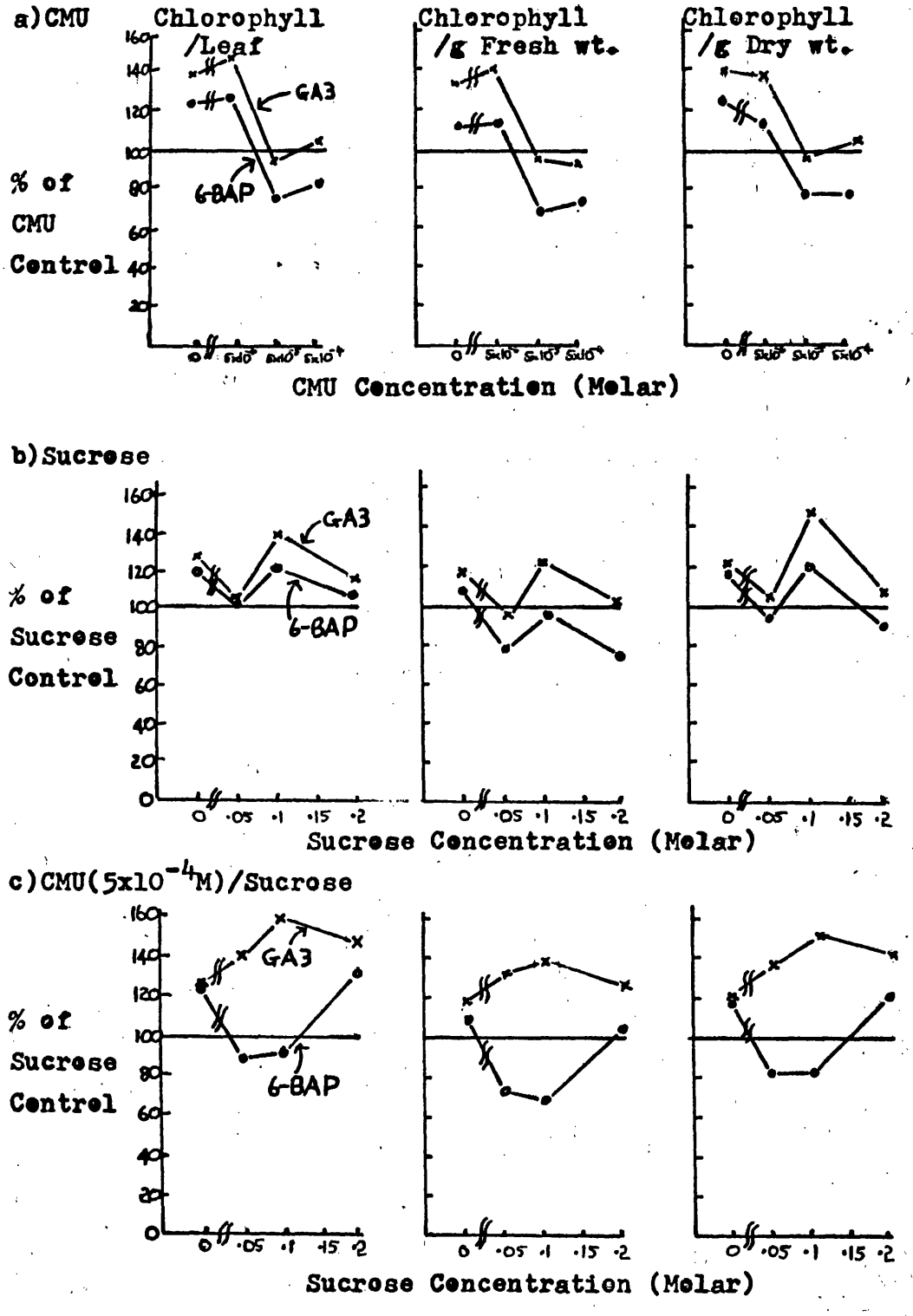
addition of sucrose caused an increased chlorophyll level in the whole leaf at all three concentrations (0.05, 0.1 and 0.2M) but on a fresh weight and dry weight basis, 0.1 and 0.2M sucrose were inhibitory, while 0.05M sucrose resulted in a 15% increase. In the presence of CMU (5×10^{-4}M), sucrose promoted the chlorophyll level per leaf considerably. The pattern of response differed from that of sucrose alone, in that the maximum effect was obtained with 0.1M and 0.2M sucrose. Again, the dry weight and fresh weight increases were sufficiently large to reduce this increase and result in levels equal to or less than that of the control. These results are in agreement with those of Klein and Neumann, (1966) and Dodge *et al*, (1971).

The data for hormone effects in Figure 17 were calculated as percentage changes of the chlorophyll content of the corresponding control treatment with CMU, sucrose and CMU/sucrose and not of the untreated control, e.g. GA_3/CMU (5×10^{-4}M) was expressed as a percentage of CMU (5×10^{-4}M). The control curves shown in Figure 16 have, thus been eliminated and the changes may be compared directly with those of the hormone treatment in the control system. Giberellic acid at 1.0 mg/l and 6-BAP at 5.0 mg/l were chosen as concentrations likely to elicit responses.

Treatment with CMU (5×10^{-6}M) did not reduce the effects of GA_3 or 6-BAP, but a tenfold increase in CMU concentration completely abolished any effect of GA_3. The curve for 6-BAP showed that this hormone inhibited the level of chlorophyll in the absence of photosynthesis. Since 5×10^{-6}M CMU inhibited chlorophyll synthesis to

FIGURE 17

EFFECT OF GA3(1mg/l) AND 6-BAP(5mg/l) ON CHLOROPHYLL CONTENT OF PRIMARY LEAVES IN THE PRESENCE OF CMU, SUCROSE & CMU/SUCROSE



NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

almost the same degree as the higher concentration (Figure 16) it is surprising that it did not abolish the effects of GA_3 and 6-BAP. This suggests that either a sufficient level of photosynthesis for these effects was achieved or CMU interfered in another way at concentrations of 5×10^{-5}M and above. The curves were similar for all three expressions of chlorophyll content.

When supplemented with sucrose (0.1M) the leaves responded more readily to GA_3 than 6-BAP, but the shapes of the curves were similar for both hormones with an optimum sucrose concentration of 0.1M. The 6-BAP effect on 0.1M sucrose-treated leaves was no greater than its effect on untreated leaves. At 0.05 and 0.2M sucrose, 6-BAP was much less effective. On a fresh weight basis 6-BAP was inhibitory in the presence of sucrose, thus indicating its large effect on expansion. The curve for chlorophyll per gram dry weight was similar to that for chlorophyll per leaf. Gibberellic acid was also less effective when incubated with 0.05 and 0.2M sucrose than it was in the control system, but 0.1M sucrose enhanced its activity. On a fresh weight basis this was not the case, which suggested that the stimulation could have been due to increased leaf expansion. The absence of promotion in the 6-BAP-treated leaves as a result of expansion, however, tends to argue against this. The increase elicited by GA_3 in the presence of 0.1M was even more evident on a dry weight basis than on a whole leaf basis. Thus, it was not a result of a general increase in leaf substance.

In the CMU/sucrose system, GA_3 exerted a promotive effect at all concentrations of sucrose and this was shown on whichever

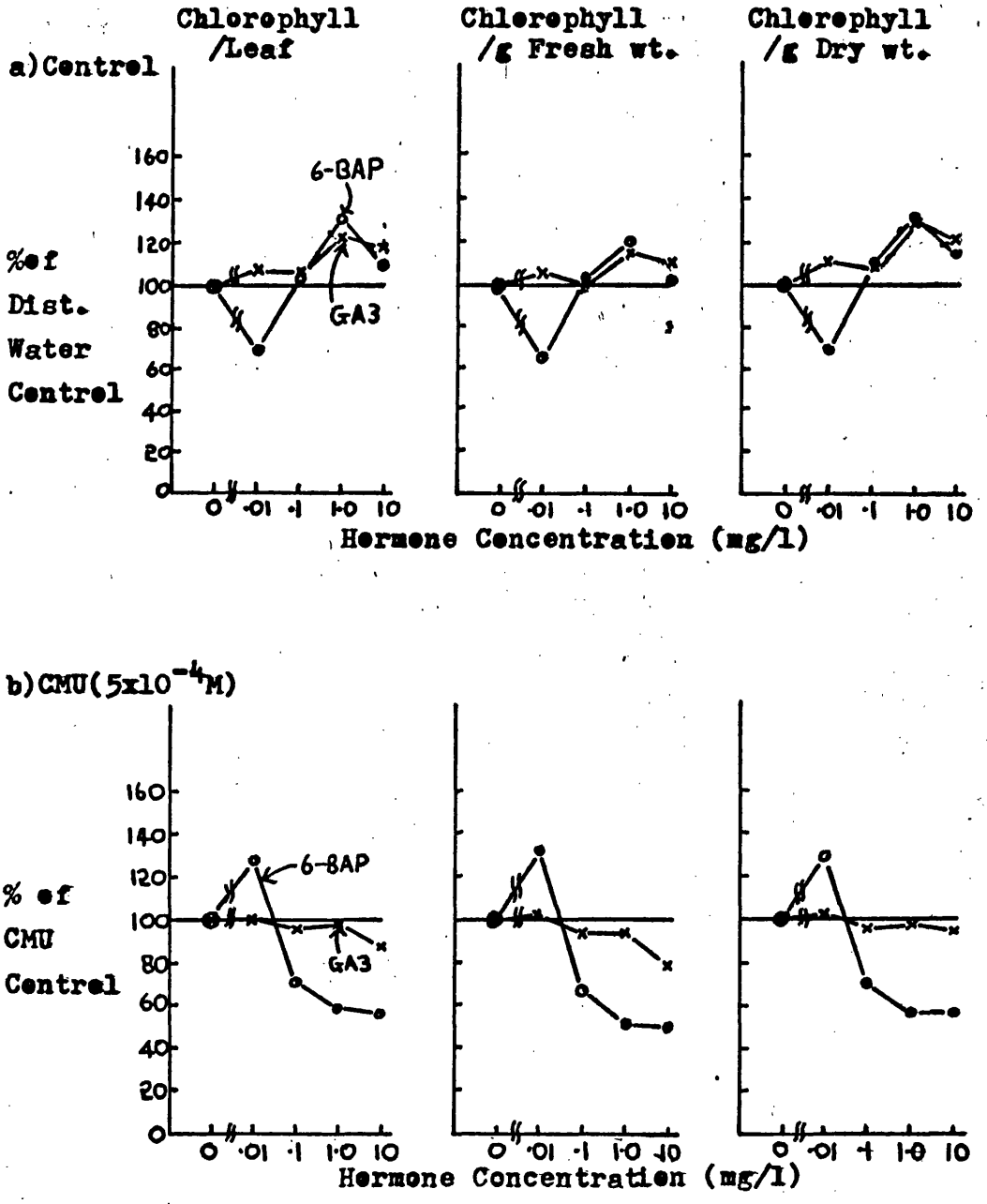
basis the chlorophyll level was expressed. 6-Benzylaminopurine was, however, inhibitory at 0.05 and 0.1M sucrose, but provoked an increase at 0.2M sucrose. This was no greater than its effect in the control system.

It may be summarized that GA_3 promoted in the presence of photosynthesis but was ineffective in its absence. The addition of exogenous substrate improved the promotion when used at certain concentration, but in the absence of photosynthesis was effective at all concentrations. On the whole these data indicate that the supply of substrate was an important factor in the promotion of chlorophyll level by GA_3. 6-Benzylaminopurine was also capable of promotion in the presence of active photosynthesis but reduced the chlorophyll level in its absence. Sucrose was not an effective aid to the 6-BAP promotion and only restored it in the absence of photosynthesis, when it was supplied at 0.2M. At the lower concentration of sucrose 6-BAP was inhibitory. Substrate level undoubtedly influenced the activity of 6-BAP but it appeared to be more related to photosynthetic substrate than to that exogenously supplied. This might indicate that 6-BAP could be involved in the promotion of photosynthetic development or its action may be dependent on the production of specific photosynthetic products. In each of these systems the general tendency for the GA_3/6-BAP curve was to follow the curve of 6-BAP alone, indicating an over-riding effect of 6-BAP [see Results 2(e)].

Figure 18 shows the effect of hormone concentration (0.01 to 10mg/l) on chlorophyll content in the four systems

FIGURE 18

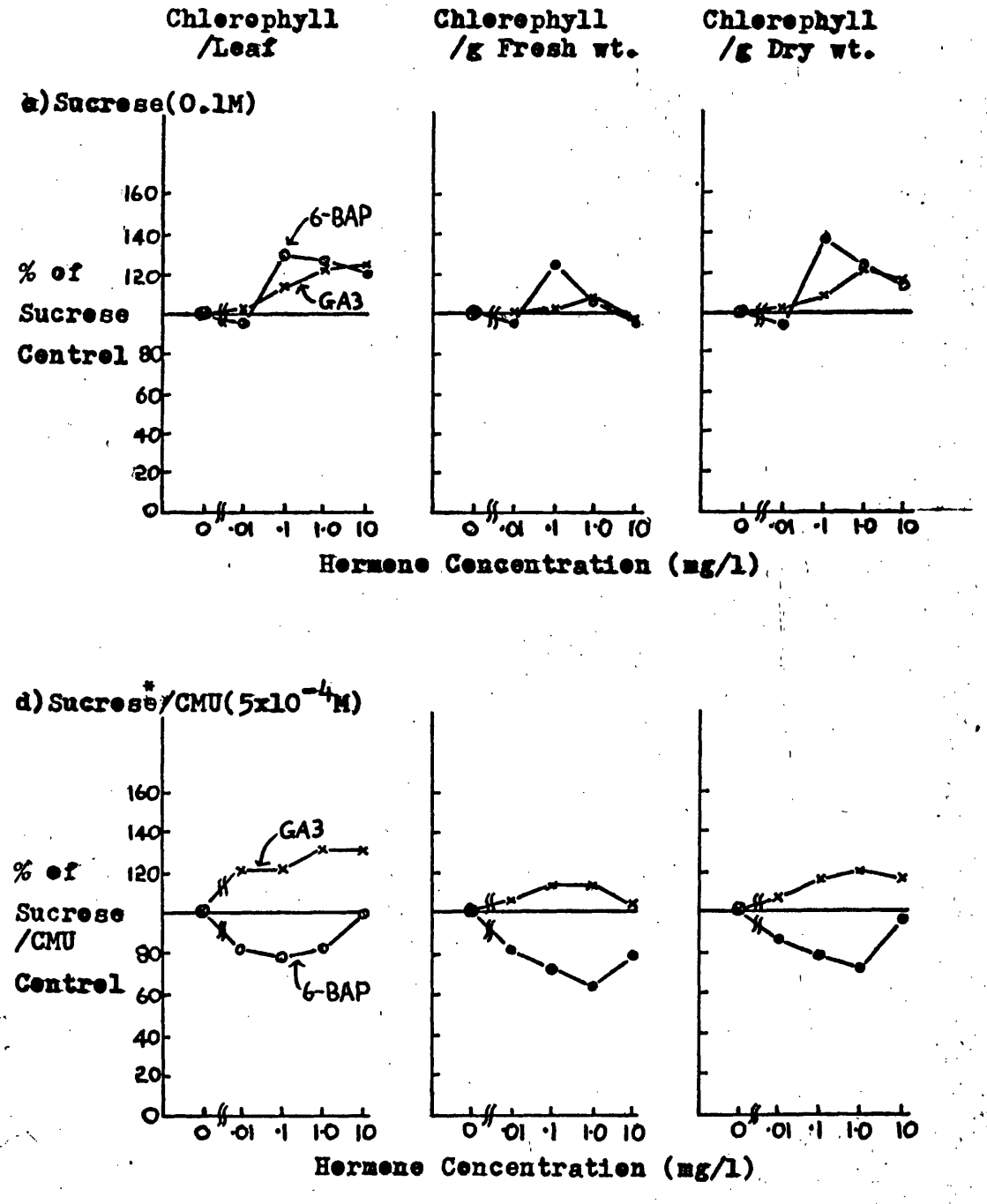
EFFECT OF GA3 AND 6-BAP CONCENTRATION ON CHLOROPHYLL CONTENT OF PRIMARY LEAVES IN THE PRESENCE OF CMU, SUCROSE & SUCROSE/CMU



NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

(continued on next page)

FIGURE 18 (Continued)



NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

* Concentration + GA3 — 0.1M
+ 6-BAP — 0.2M

previously described. They are from one experiment only and were obtained to check that the concentrations used were optimal. Both hormones were most effective in the 1.0 mg/l range in the control system. At 0.01 mg/l 6-BAP was inhibitory. This agrees with results from the factorial experiments on the interaction of GA_3 and 6-BAP [see Results 2(e)] which suggest a diphasic response to 6-BAP.

As shown by the previous set of results, GA_3 was ineffective in the presence of CMU (5×10^{-4}M) and altering its concentration had no effect. 6-Benzylaminopurine was inhibitory at concentrations of 0.1 to 10 mg/l and showed a small increase in inhibition with increasing 6-BAP concentration. At 0.01 mg/l, 6-BAP promoted the chlorophyll level, thus the 6-BAP curve was almost a mirror image of the results obtained in the control system.

When sucrose was added, GA_3 showed increasing promotion with increasing concentration up to 1.0 mg/l. On a chlorophyll per leaf basis, 6-BAP promoted equally well at concentrations of 0.1, 1.0 and 10 mg/l but on fresh weight and dry weight bases 0.1 mg/l 6-BAP was the most effective. This change of optimum may explain the poor response of 6-BAP in the presence of sucrose as shown in Figure 17. It also indicates that the lower 6-BAP concentration promoted chlorophyll synthesis more specifically than the higher concentrations. These seemed to promote the general increase of leaf substance. The relationship of chlorophyll content with GA_3 concentration was very much the same in the presence of CMU/sucrose as was observed in the presence of sucrose alone, but the response to

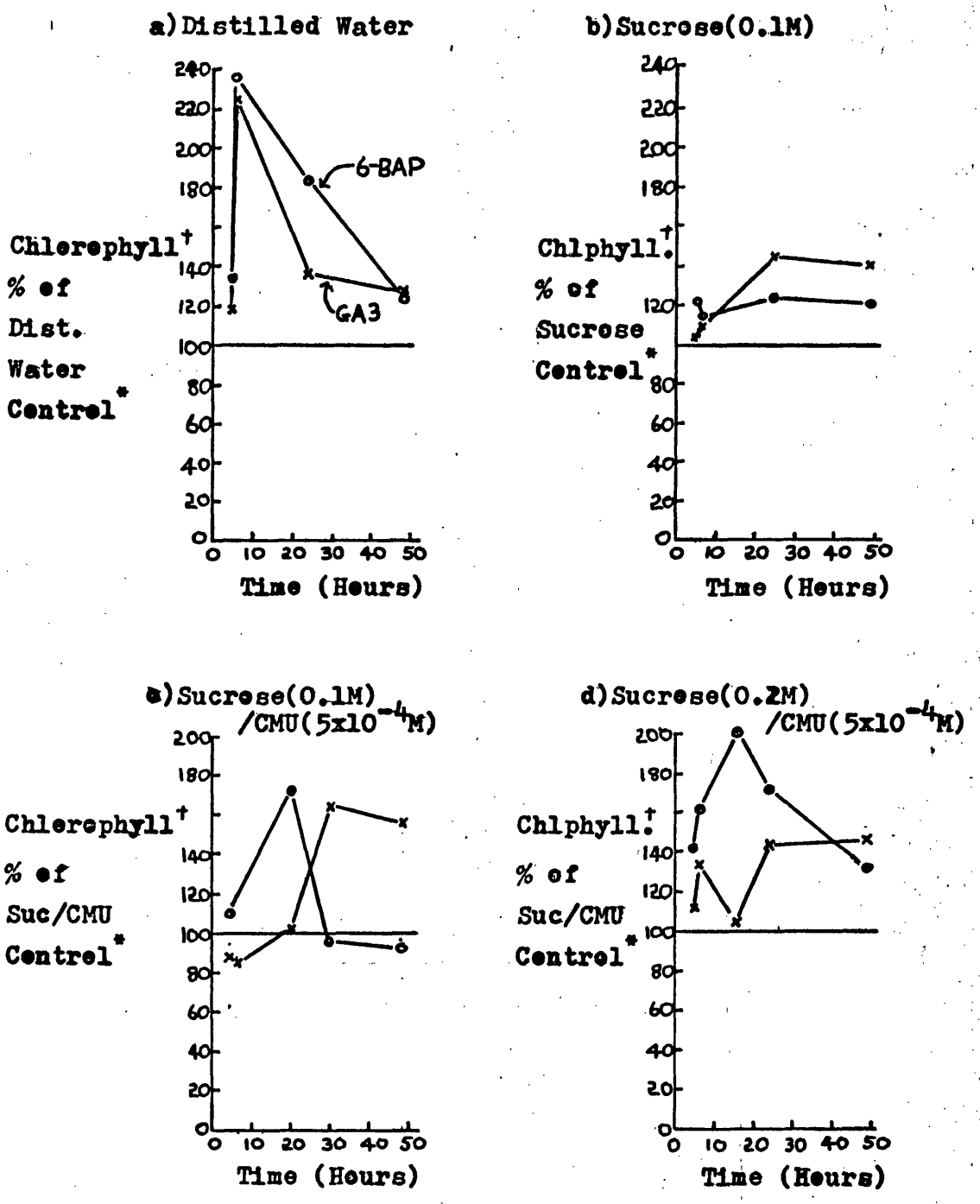
6-BAP confirmed earlier observations that it was inhibitory when photosynthesis was inhibited. Only 10 mg/l 6-BAP was not inhibitory and the chlorophyll level in the leaves treated with this was similar to that of the control. These data confirm that the concentrations used in other experiments were optimum, with the exception of 6-BAP in the presence of sucrose (0.1M) where a concentration lower than 5 mg/l may have been preferable.

The time courses (Figure 19) for the development of the effects of GA_3 (1 mg/l) and 6-BAP (5 mg/l) were investigated in the control system, in the presence of sucrose (0.1M) and CMU (5×10^{-4}M) /sucrose (0.1 and 0.2M).

In the control system, both hormones were effective after about 5 hours and a maximum response was elicited at 7 hours. After this, the effect declined to about 25% at 48 hours. The decline was more rapid in the GA_3-treated than in the 6-BAP-treated leaves. When the leaves were incubated on sucrose there were no large hormone-induced chlorophyll increases in the early stages of illumination. Gibberellic acid promotion became evident between 5 and 10 hours and increased to 43% during the first 24 hours. Subsequently it remained constant. 6-Benzylamonopurine promoted after 5 hours illumination and remained constant at 23% throughout the time course. When sucrose (0.1 and 0.2M) and CMU (5×10^{-4}M) were present in the incubation medium, 6-BAP exhibited a large promotion after about 16 hours. This then declined to the control level in 0.1M sucrose and more gradually to 32% increase in 0.2M sucrose. Gibberellic acid had no enhancing effects until after

FIGURE 19

TIME COURSE OF GA3(1mg/l) AND 6-BAP(5mg/l) EFFECTS IN THE PRESENCE OF NO SUBSTRATE, SUCROSE & SUCROSE/CMU



NOTE: Illuminated at 1000 lux : 5-day old leaves

* At their respective times during illumination

† Chlorophyll/Leaf

24 hours in CMU/0.1M sucrose. From this time the effect was maintained at approximately 60% for the duration of the incubation. In CMU/0.2M sucrose its promoting effect appeared earlier (7 hours) and increased to only 45% during the illumination period. The final level of GA_3 promotion was consistent with that of earlier results (Figure 17).

In the absence of photosynthesis 6-BAP was ineffective after 5 hours illumination and inhibitory after 48 hours, as in earlier experiments (Figures 11 and 18). The effect of GA_3 at these times was the converse of this. The trends of these curves corresponded to those in the CMU/sucrose system.

It may be concluded that both hormones affected chlorophyll synthesis soon after the onset of photosynthesis in the control system, (see Results Section 4). When sucrose was added these large increases were not observed, because the roles of the hormones at this stage had to some extent been replaced. The later level of promotion was, however, still evident. The inhibition of photosynthesis in the presence of sucrose also eliminated the early promotion of GA_3. 6-Benzylaminopurine however, induced a large increase in chlorophyll level. The effects later in the time course were dependent on the level of sucrose used.

As suggested previously, the action of GA_3 appeared to depend on the presence of a supply of substrate and the results are compatible with this idea. The early promotion by 6-BAP in CMU/sucrose is also consistent with the suggestion that 6-BAP may be involved in photosynthetic development rather than general

utilization of substrate. The absence of an early peak in the sucrose treatment does, however, contradict this. It can only be assumed that in this system there is saturation of substrate supply and this can replace the hormonal activity.

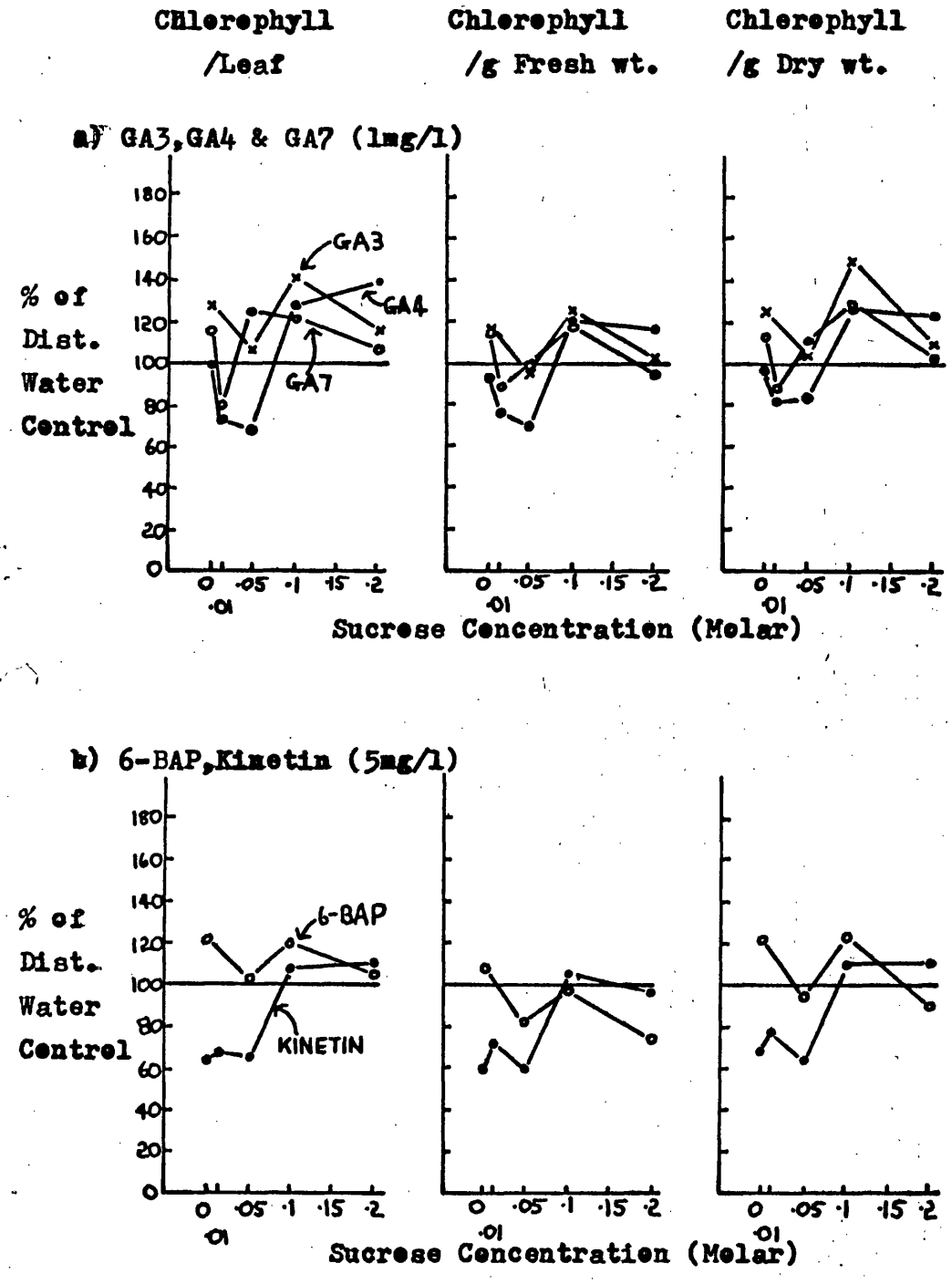
c) Effect of other Growth Hormones on Chlorophyll Synthesis

For comparison with GA_3 and 6-BAP, two other gibberellins (GA_4 and GA_7) and one other cytokinin (kinetin) were tested. The results of the experiment are illustrated in Figure 20. Of the gibberellins, GA_7 (1 mg/l) promoted when supplied alone but GA_4 (1 mg/l) was without effect. When sucrose was added, GA_7 was inhibitory at 0.01M but promoted at 0.05, 0.1 and 0.2M. The promotion was only slightly higher than in the control system at 0.05 and 0.1M and was much lower at 0.2M sucrose. On a dry weight basis, promotion was maximal at 0.1M sucrose. Gibberellin GA_4 was also inhibitory at low sucrose concentration (0.01 and 0.05M), but exhibited considerable promotive effects at 0.1 and 0.2M. These were also present on a fresh weight and dry weight basis. It appears therefore that GA_4 required the presence of sucrose for its activity while GA_7 exhibited activity with and without sucrose. Since these changes were present on whichever basis chlorophyll was expressed they reflect changes in both the total and proportion of chlorophyll. Neither GA_4 or GA_7 was as active as GA_3.

Kinetin (5 mg/l) was inhibitory when supplied alone and with 0.01 and 0.05 sucrose, but this was offset by 0.1 and 0.2M sucrose. The only indication of a similarity with 6-BAP was a

FIGURE 20

EFFECT OF GA4, GA7 AND KINETIN ON CHLOROPHYLL CONTENT OF PRIMARY LEAVES ± SUCROSE



NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

suggestion of an increase at the higher sucrose concentrations. The different responses observed between the three gibberellins and the two cytokinins may, of course, have been due to a concentration differential which was not fully explored.

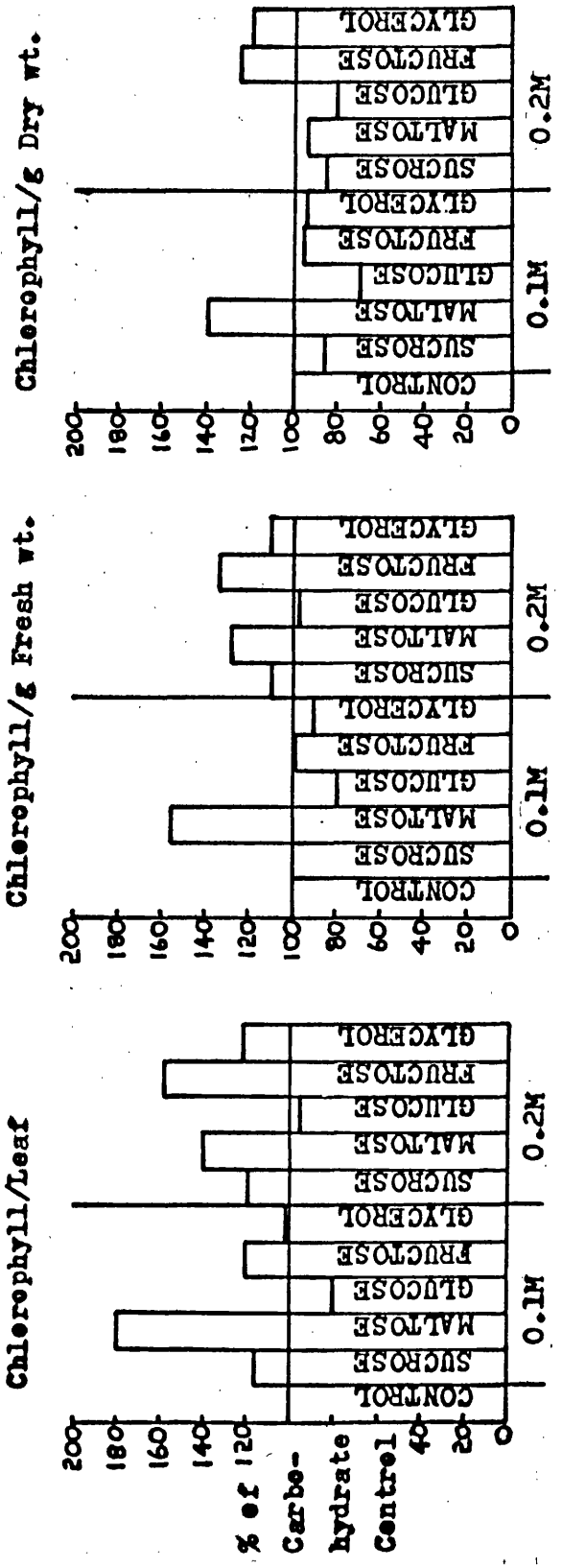
d) Carbohydrate Source and Hormone Response

Both hormones, particularly GA_3, responded to the presence of exogenously supplied sucrose. The nature of substrate supply was investigated further by examining the specificity of this response. In addition to the carbohydrates shown in Figure 21, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate were examined. These sugar-phosphates caused complete inhibition of chlorophyll synthesis at 0.1M. The effect of inorganic phosphate on chlorophyll synthesis was subsequently investigated, and complete inhibition was found at 0.1 and 0.2M. When supplied at 0.05M it resulted in only 60% inhibition. These results confirm those of Wolff and Price (1960).

The histograms in Figure 21(a) show the effects of the various substrates (sucrose, maltose, glucose, fructose and glycerol) on chlorophyll per leaf, per gram fresh weight and per gram dry weight at concentrations of 0.1 and 0.2M. The amount of chlorophyll per leaf was promoted by sucrose, maltose and fructose at 0.1M while glucose was inhibitory and glycerol ineffective. The largest increase was elicited by maltose. This was four times those elicited by fructose and sucrose. At 0.2M, the effect of maltose was considerably less, though still larger than that of sucrose.

FIGURE 21

EFFECT OF GA3 AND 6-BAP ON CHLOROPHYLL CONTENT OF PRIMARY LEAVES IN THE PRESENCE OF VARIOUS CARBOHYDRATES



a) Effect of carbohydrate alone

(continued on next page)

b) Effect of GA3 (1mg/l)

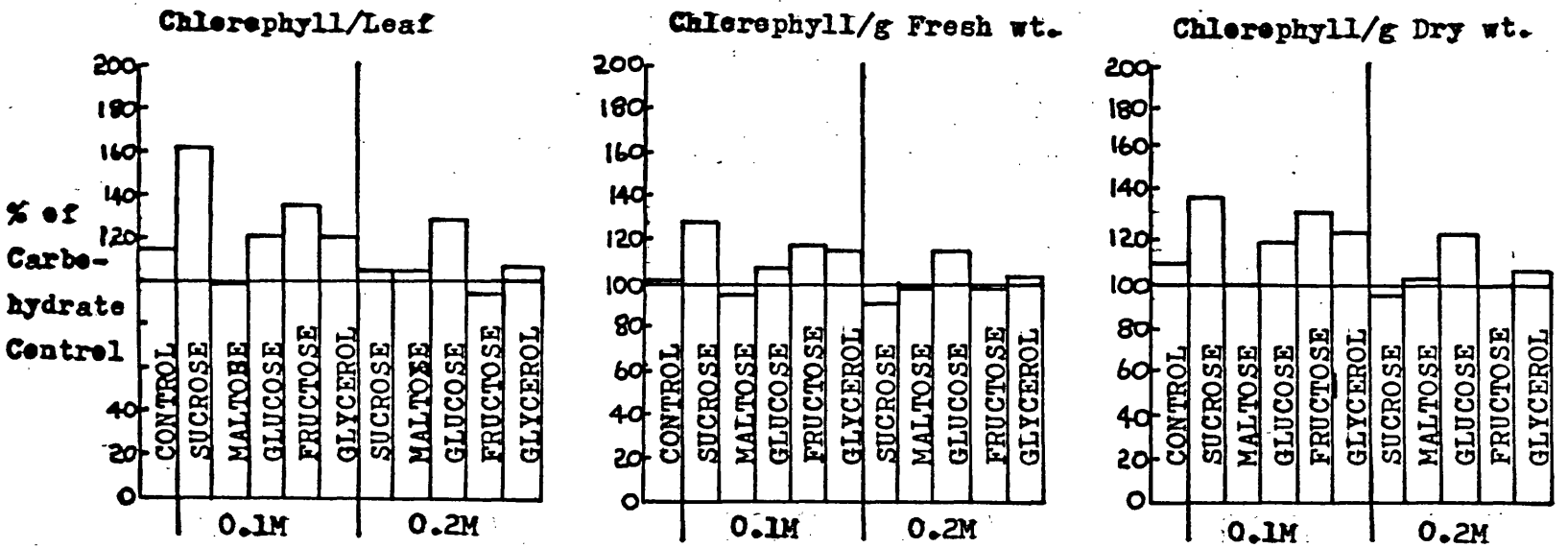


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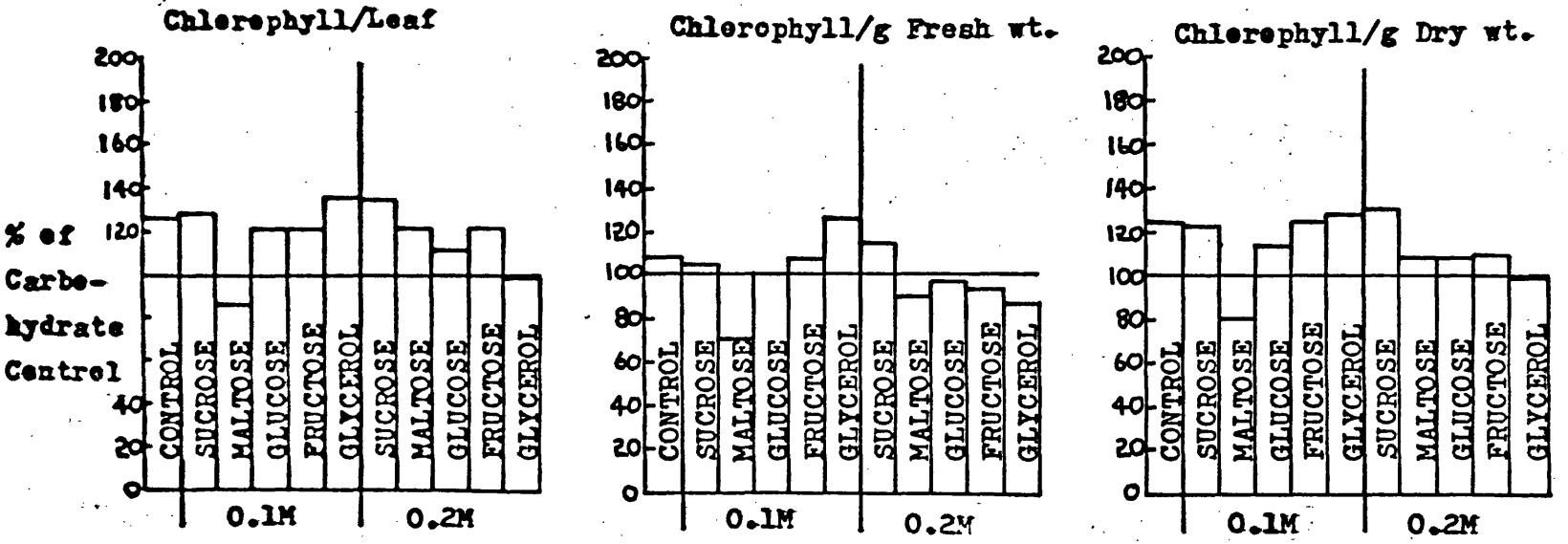
FIGURE 21 (Continued)

c) Effect of 6-BAP (5mg/l)



NOTE: 1. Leaves illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

FIGURE 21 (Continued)

Fructose (0.2M) gave the highest promotion and glycerol (0.2M) was as effective as sucrose. Glucose again inhibited, but to a much lesser degree. On a fresh weight basis, only maltose increased the chlorophyll level at 0.1M concentration, but at 0.2M the changes were the same as for chlorophyll per leaf. When dry weight changes were taken into consideration, all of the substrates were inhibitory with the exception of maltose (0.1M) fructose (0.2M) and glycerol (0.2M). It is surprising that maltose, which is composed of two glucose units should be so effective while glucose itself was inhibitory. The effect of maltose may be associated with the disaccharide nature of the molecule. The intermediate position of sucrose in relation to the fructose and glucose levels was however in agreement with their individual effects. Undoubtedly the fructose moiety is an active part of sucrose in its effect on chlorophyll synthesis. The small effect exerted by glycerol was expected since, as a triose sugar, it represented less available energy than the others. Wolff and Price (1960) measured protochlorophyllide production in leaves of *P. vulgaris* three hours incubation on various substrates and observed increases due to glycerol, glucose, fructose, sucrose and maltose at both 0.1M and 0.2M. Of these, the least effective were glycerol and fructose. Glucose was as effective as either of the disaccharides. Comparison of the two sets of data suggests that the inhibitory effects which occur after 48 hours are the result of interference with metabolic activities which are not present at such an early stage as 3 hours. Harris and Naylor (1968) also reached the conclusion that some compounds may initially stimulate chlorophyll synthesis. They, too

found that in greening tobacco leaves, a concentration of 0.02M glucose was stimulatory while 0.2M glucose was not.

The GA_3 promotion of chlorophyll synthesis was enhanced most by 0.1M sucrose. Of the other carbohydrates at 0.1M only fructose was particularly effective while glucose and glycerol made little difference and maltose erradicated its promotion. At 0.2M only glucose effectively increased GA_3 promotion; the other carbohydrates were all inhibitory. The same pattern was evident on fresh weight and dry weight basis. The efficacy of glucose and fructose is consistent with the positive effect exerted by sucrose.

Only 0.1M glycerol and 0.2 sucrose improved the 6-BAP-induced promotion on a chlorophyll/leaf basis and on a dry weight basis this was less evident, so the effect seemed to be through a general increase in leaf dry matter. Glycerol was more effective when the results were expressed on a fresh weight basis, indicating that the 6-BAP induced expansion was either counteracted or chlorophyll synthesis was stimulated to keep in step. Of the other carbohydrates, fructose and maltose had no effect while glucose was inhibitory.

Since the metabolism of all these compounds is closely related it might be expected that their effects on chlorophyll synthesis would correlate with the amount of energy available and show an interaction with the osmolarity at which they were supplied. This was not the case, and, thus, indicates that there were certain specific effects. Since both maltose and sucrose produce glucose

units on hydrolysis, they also might be expected to inhibit chlorophyll synthesis. One may conclude that as this was not observed, there was a direct inhibition due to the presence of exogenously supplied glucose and this did not occur as a result of endogenously produced glucose. Oochiai and Hase (1970) found that glucose suppressed chlorophyll synthesis in *Chlorella prototheccoides*. This was also observed for fructose, galactose, glycerol and acetate and so may be considered different.

In mung bean leaves sucrose might have been expected to have been as effective as maltose, particularly as fructose alone exerted a promotive influence. It is possible that exogenously supplied sucrose also inhibited chlorophyll synthesis but the presence of the fructose unit counteracted this. Sucrose suppression of chlorophyll synthesis has been observed in carrot callus tissue (Edelman and Hanson, 1971).

The influence of GA_3 seemed to be associated with the presence of the fructose molecule. It is, true however that glucose at 0.2M was also effective. These results were contradictory since maltose inhibited the GA_3 promotion. The explanation of this depends on the precise fate of the sugars inside the leaf. They may either be metabolised through the E.M.P. pathway or incorporated into starch. The data of Wolff and Price (1960) show that addition of sucrose resulted in increased starch production. If starch production is a feature of carbohydrate treatment of mung bean leaves then GA_3 may be effective through its subsequent hydrolysis as it is in germinating seeds (Varner, 1964). Since

maltose units are possibly one of the initial breakdown products of starch (Akazawa, 1965) the GA_3 activity is bypassed. This argument could be made for any of the carbohydrates and as already stated depends on the degree to which each was incorporated into starch reserve. Certainly maltose itself is not reversibly transformed into starch (Akazawa, 1965) but by metabolism to glucose this becomes distinctly probable.

The results for 6-BAP illustrate again the lesser dependence of the action of this hormone on the presence of excessive substrate.

The chlorophyll *a/b* ratios for leaves treated with hormones and substrate revealed no discernible differences between treatments.

e) The Interaction of GA_3 and 6-BAP in Controlling Chlorophyll Synthesis

Testing GA_3 and 6-BAP in various substrate regimes has revealed characteristic properties in their effects on chlorophyll synthesis. The hormones may be further characterized by examining them in combination. This may emphasize the extent to which they are specific and how they may interact in the whole plant to play a part in controlling chloroplast development.

The effects of GA_3 and 6-BAP were investigated with $3\frac{1}{2}$, 5 and 7 day old etiolated leaves during a 48 hour illumination period. The results, on a chlorophyll per leaf basis, are shown in Table 1. Tenfold dilution ranges (0.001 - 10 mg/l) of the hormones were used

TABLE 1

INTERACTION OF GA3 AND 6-BAP AND THE EFFECT ON CHLOROPHYLL SYNTHESIS

a) 3½ day-old leaves

6-BAP Concn. (mg/l) \ GA3 Concn. (mg/l)	0	.001	.01	.1	1.0	10
0	100	95	95	86*	81*	112*
.001	85*	88	83*	92	92	100
.01	81*	81*	76*	81*	95	110
.1	105	114	110	124**	124**	105
1.0	85*	94	113	106	119	125*
10	79*	81	94	100	113	113

b) 5 day-old leaves

6-BAP Concn. (mg/l) \ GA3 Concn. (mg/l)	0	.001	.01	.1	1.0	10
0	100	110	110	103	107	117***
.001	84*	77**	86**	77*	83*	94
.01	71**	49***	56**	56*	90	95
.1	94	87	85	105	100	103
1.0	95	109	120	129*	143**	126*
5.0	112	119	169***	174***	169***	156***
10	105	111	131**	120	117	129*

Significance

* 5.0%

** 1.0%

*** 0.1%

c) 7 day-old leaves

6-BAP Concn. (mg/l) \ GA3CConcn. (mg/l)	0	.001	.01	.1	1.0	10
0	100	94	94	98	100	111
.001	75**	90	92	92	80*	98
.01	81*	94	97	90	97	106
.1	85*	92	76**	76**	102	93
1.0	92	98	102	76*	95	105
10	91	92	80*	105	95	105

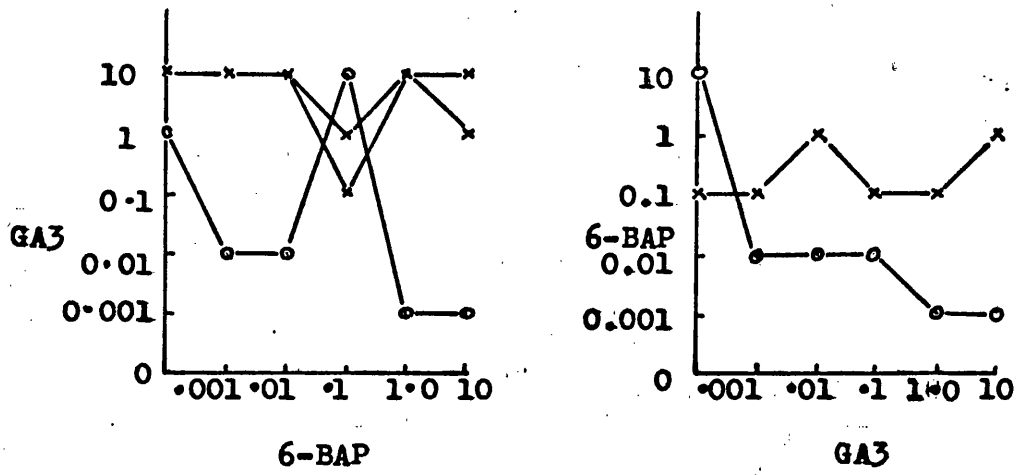
Note. Results represent chlorophyll (mg/leaf) expressed as % distilled water control.

and in addition, in 5 day old leaves a 5 mg/l concentration of 6-BAP was included. Each result is the average of at least four replicates of samples containing ten leaves. The significant differences indicated in Table 1 were determined by the Student *t* test and are the differences as compared with the untreated controls. In 3½ day old leaves promotion of the chlorophyll level in the presence of the individual hormones was observed only at a concentration of 10 mg/l GA_3, while inhibition occurred at 0.1 and 1.0 mg/l GA_3 and at .001, .01, 1.0 & 10 mg/l 6-BAP. All of these were significant at the 5% probability level. In combination, a peak of promotion was exhibited at 0.1 mg/l 6-BAP and 0.1 - 1.0 mg/l GA_3 with fairly high increases at 1.0 mg/l 6-BAP and 1.0 - 10 mg/l GA_3. When the lower concentrations were combined, a decreased level of chlorophyll was observed, the greatest inhibition being at .01 mg/l 6-BAP and .01 mg/l GA_3. Increasing the GA_3 concentration appeared to offset the inhibitory effect of 6-BAP and at the higher 6-BAP concentrations the relationship showed signs of synergism. As 6-BAP was increased in concentration the two hormones became more effective but this began to decrease again at 10 mg/l GA_3. The relationship of hormone concentration (Figure 22) showed that at concentrations where 6-BAP alone was inhibitory the highest concentration of GA_3 gave most effective reversal, but at the 6-BAP concentration of least inhibition (0.1 mg/l), the optimum GA_3 concentration decreased from 10 mg/l to 0.1 - 1.0 mg/l. The 6-BAP concentration which resulted in the highest and lowest percentage changes in chlorophyll content at a given GA_3 concentration remained fairly constant at 0.1 - 1.0 mg/l and 0.001 - 0.01 mg/l respectively. This contrasted with the

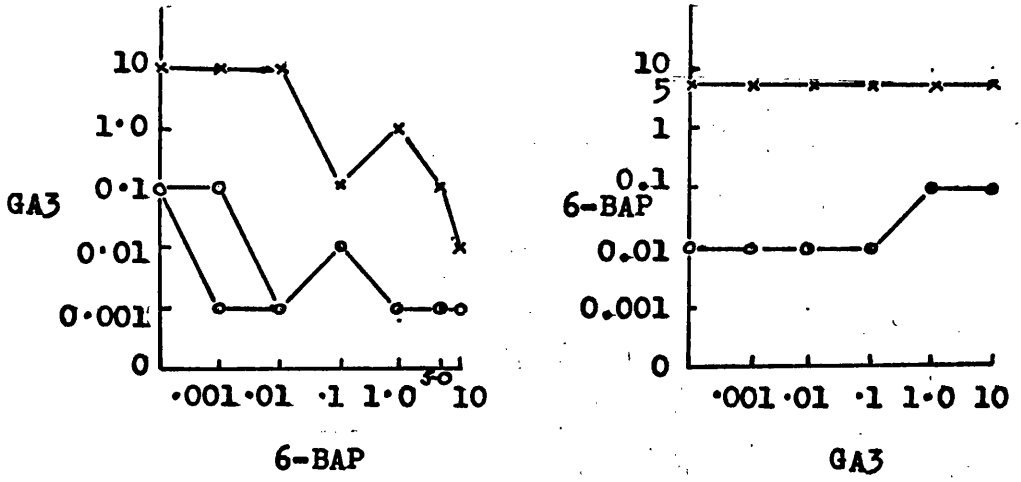
FIGURE 22

CONCENTRATION* RELATIONSHIP BETWEEN GA3 AND 6-BAP

a) 3½ day old leaves



b) 5 day old leaves



×—× HIGHEST VALUE

o—o LOWEST VALUE

* Concentration: mg/l

trends observed for GA_3 concentration against 6-BAP concentration which showed that optimum GA_3 concentration altered in accordance with the 6-BAP effect. The general trend indicated that the hormones were mutually antagonistic to their inhibitions and that at certain concentrations where synergistic promotion occurred the effect was due to a change in response of the chlorophyll level to 6-BAP. The antagonism was supported by the observation that the concentration where the hormones alone resulted in the highest value (10 mg/l GA_3 and 0.1 mg/l 6-BAP) no increase was observed when they were applied together. The data for fresh weight increases caused by the hormones did not show corresponding changes to the data for chlorophyll increases. It was concluded, therefore that changes in leaf expansion were an unlikely cause of the changes in chlorophyll level.

In five day old etiolated leaves no inhibition of the chlorophyll level was caused by GA_3 and promotion was significant at 10 mg/l GA_3 at the 0.1% level of probability. Significant inhibitions were however observed at 0.001 and 0.01 mg/l 6-BAP. The level of chlorophyll gradually increased with increasing 6-BAP concentration to 5 mg/l. In combination with 6-BAP, GA_3 at high concentration (10 mg/l) counteracted the 6-BAP inhibition, but a lower concentrations it appeared to enhance the inhibition, particularly at 0.01 mg/l 6-BAP. At 1.0, 5.0 and 10 mg/l, 6-BAP caused considerable promotion and a significant synergistic effect was observed. This reached a maximum at 5.0 mg/l 6-BAP and 0.1 mg/l GA_3. The 6-BAP concentration peak showed a greater specificity for concentration than the GA_3 peak which was spread fairly evenly from 0.01 to 1.0 mg/l. The concentration relationship (Figure 22) of the two hormones revealed that GA_3 at

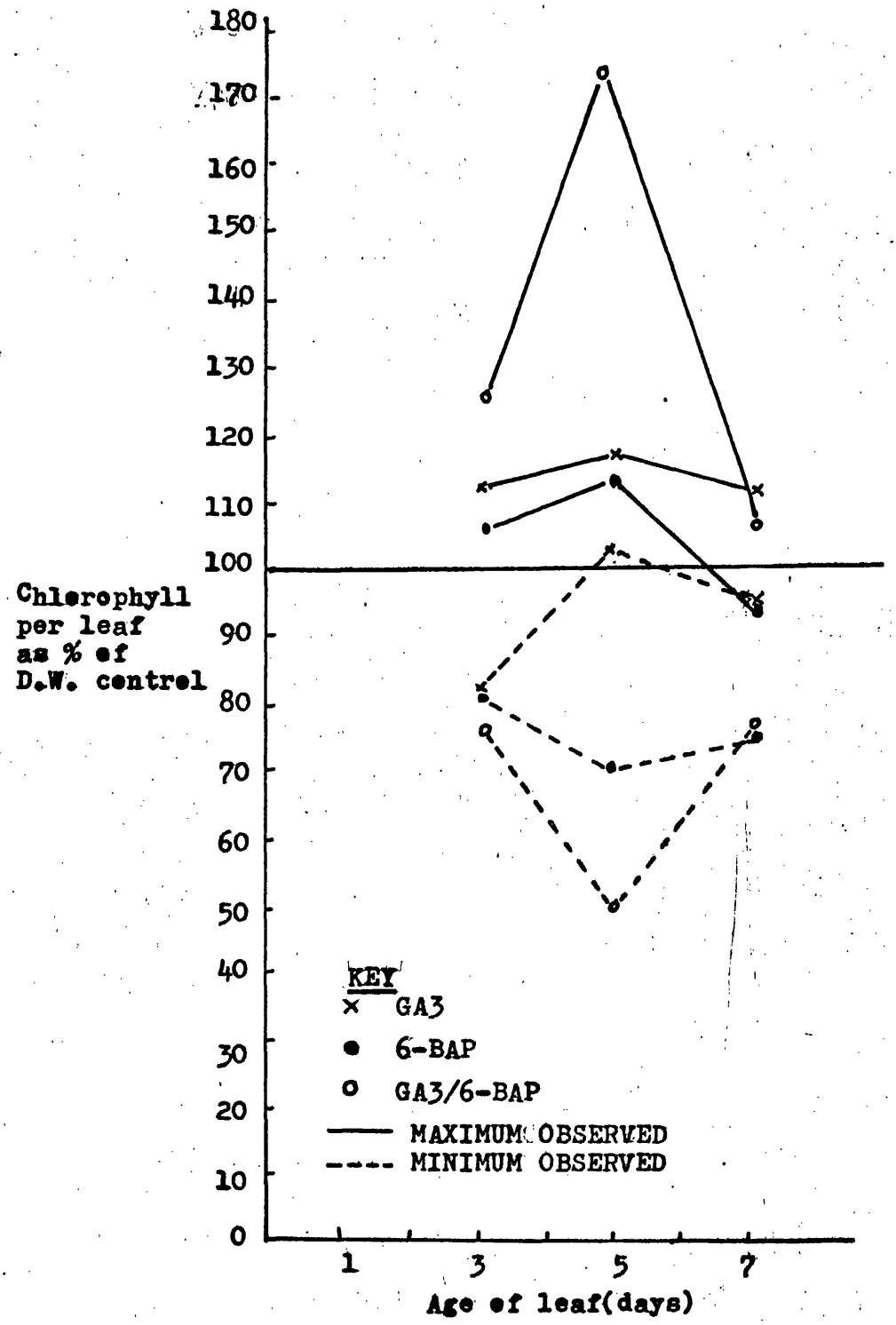
high concentration (10 mg/l) reversed the 6-BAP inhibition at low concentration. Once again the synergistic promotion occurred in the optimum concentration for 6-BAP alone, but the level of GA_3 necessary was less than 10 mg/l which was the optimum level when applied alone. As the concentration of 6-BAP was increased within the 1.0 to 10 mg/l range, the effective concentration of GA_3 gradually decreased, suggesting that an increase in sensitivity to GA_3 took place. The concentrations of 6-BAP giving the highest and lowest values remained very constant at 5.0 mg/l and 0.01 - 0.1 mg/l. In ageing from $3\frac{1}{2}$ to 5 days, the leaves became responsive to higher concentrations of 6-BAP but showed the same diphasic trend. The reversal of 6-BAP inhibition by high GA_3 concentration was still present but the antagonism between the optimum concentrations of the two hormones alone was not evident. The fresh weight data were again of a different pattern to the chlorophyll data.

After 7 days etiolated growth very little effect could be discerned when the hormones were applied to the leaves. The only significant differences were inhibitions which appeared to be due to the presence of 6-BAP at 0.001, 0.01 and 0.1 mg/l. Since the 6-BAP optimum concentration for the synergistic promotion increased from 0.1 to 5 mg/l during the time the leaves aged from three to five days, it is possible that seven day old leaves may have responded to higher 6-BAP concentrations.

The age response is summarised in Figure 23. The graph shows the maximum and minimum effects obtained with the three main treatments (GA_3, 6-BAP and GA_3/6-BAP) regardless of the concentrations

FIGURE 23

EFFECT OF AGE OF LEAF ON RESPONSE OF CHLOROPHYLL LEVEL TO GA3, 6-BAP AND GA3/6-BAP.



used. The order of the response (i.e. 5, 3 and 7 days) was inversely related to the total amount of gibberellins assayed in the leaves (see Results Section 3).

The results from five day old leaves indicated that there was one site of inhibition which was stimulated by lower concentrations of 6-BAP and a site of promotion operated in the 5 mg/l range. The inhibitory site when in combination with the GA_3-operated stimulatory site resulted in an enhanced inhibition while the interaction of the stimulatory sites was synergistic. This situation also existed in 3½ day old leaves, but in addition there was an inhibitory site operated by low concentrations of GA_3. This latter site seems to have disappeared as the leaf developed.

The maximum promotions elicited by combination of GA_3 and 6-BAP in five day old leaves were examined under various substrate regimes and generally followed the curves for 6-BAP alone see Results Section 2(d) .

f) Effect of GA_3/6-BAP Under Differing Regimes of Substrate Supply

A new batch of beans was examined for the effect of combinations of GA_3 and 6-BAP on the chlorophyll content of the primary leaves under the same conditions as the previous experiment (Table 2). This time, however, concentration ranges of 0.1 to 10 mg/l of each hormone were used and these were tested in four

TABLE 2

EFFECT OF INTERACTION OF GA3 AND 6-BAP ON CHLOROPHYLL SYNTHESIS IN VARIOUS SUBSTRATE REGIMES

a) Control (Distilled water)

6-BAP Concn. (mg/l)	GA3 Concn.(mg/l)				GA3 Concn.(mg/l)			
	0	.1	1.0	10	0	.1	1.0	10
0	100	110	114	124	100	106	110	115
.1	103	117	141	140	109	122	145	136
1.0	130	125	114	116	131	123	115	122
10	108	125	132	132	116	122	131	136

b) 0.1M Sucrose

6-BAP Concn. (mg/l)	GA3 Concn.(mg/l)				GA3 Concn.(mg/l)			
	0	.1	1.0	10	0	.1	1.0	10
0	100	129	146	146	100	132	132	152
.1	116	140	146	137	136	138	138	136
1.0	125	128	129	163	124	115	121	149
10	120	106	132	165	115	126	150	178

c) 0.1M Sucrose + CMU ($5x10^{-4}$)

6-BAP Concn. (mg/l)	GA3 Concn.(mg/l)				GA3 Concn.(mg/l)			
	0	.1	1.0	10	0	.1	1.0	10
0	100	97	111	107	100	103	108	121
.1	96	121	96	120	80	138	95	122
1.0	111	119	117	114	114	119	109	113
10	101	109	94	136	99	114	95	114

d) 0.2M Sucrose + CMU ($5x10^{-4}$)

6-BAP Concn. (mg/l)	GA3 Concn.(mg/l)				GA3 Concn.(mg/l)			
	0	.1	1.0	10	0	.1	1.0	10
0	100	85	106	102	100	77	99	102
.1	111	141	93	108	105	120	77	92
1.0	117	89	95	86	119	80	80	80
10	92	91	127	104	82	84	101	96

Chlorophyll/leaf | Chlorophyll/mg dry wt.

Note : Results are expressed as %ages of the respective regime control.

systems (Table 2):

a) under normal greening conditions

b) incubated on sucrose (0.1M)

c) incubated on CMU/sucrose (0.1M)

d) incubated on CMU/sucrose (0.2M)

Two concentrations of sucrose were tested in the sucrose/CMU system because it was found that 6-BAP responded to 0.2M sucrose in the presence of CMU while GA_3 was more effective with 0.1M sucrose (see Figure 17). In the control system (Table 2) GA_3 and 6-BAP gave a promotion at 10 mg/l and 1.0 mg/l respectively. This was similar to the results for the previous batch of seeds. Their interaction, however, was somewhat altered. In the previous beans, GA_3 was most effective at the optimum 6-BAP concentration. In this batch the most positive interactions were at sub- and supra-optimal concentrations of 6-BAP. When 6-BAP was optimal, GA_3 antagnonised its promotion. At 0.1 mg/l 6-BAP there was an indication of a synergism but at 10 mg/l the response was additive. The results were essentially the same whether expressed as chlorophyll per leaf, per gram fresh weight or per gram dry weight, except that on a fresh weight basis the percentage increases were smaller. As in the previous factorial experiment the tendency was for the interaction to be governed by the response to 6-BAP concentration.

The addition of sucrose was tried in order to determine whether the level of substrate may have been a regulating factor in the response and whether it altered the relationship of the two hormones. The highest increases were obtained at 1.0 and 10 mg/l

6-BAP in the presence of 10 mg/l GA_3. It may be concluded the relationship did change since these concentrations were also the most effective when applied alone. In some of the combinations of lower concentrations the increases were no larger than those elicited by the individual hormones, thus antagonism had undoubtedly existed, but the concentration response had altered. The maximum responses to the individual hormones varied according to the way the results were expressed.

In 0.1 M sucrose/CMU, on a chlorophyll per leaf basis the results were erratic. There appeared to be a suggestion of synergistic response at 0.1 mg/l GA_3 with all concentrations of 6-BAP and also at 10 mg/l GA_3 with 0.1 and 10 mg/l 6-BAP. When expressed on a dry weight basis, only the response at 0.1 mg/l GA_3 and 6-BAP (0.1 - 10 mg/l) was synergistic. On a fresh weight basis, most of the treatments were inhibitory. In 0.2M sucrose/CMU the chlorophyll level was promoted by combinations of 0.1 mg/l GA_3 with 0.1 mg/l 6-BAP and 1.0 mg/l GA_3 with 10 mg/l 6-BAP when expressed as chlorophyll/leaf. When based on dry weight, only the increase at 0.1 mg/l of GA_3 and 6-BAP was evident and this was synergistic.

It may be concluded that in this batch of beans, the relationship of GA_3 and 6-BAP exhibited both antagonism and synergism, as in the previous batch, but that the concentration response had altered. Addition of exogenous substrate removed any tendency for synergism at the concentrations tested, but the interaction could be additive. The removal of photosynthesis as a source of substrate lowered the general response to the hormones but did seem to reinstate a synergistic increase in chlorophyll content at a combination of the lowest concentrations.

3. Endogenous Hormones

It is advantageous to compare the effects of exogenously applied hormones with levels of hormone present in the plant and to measure the effect of artificially altering the internal hormone level or interfering with endogenous hormone activity. The importance of the level of endogenous gibberellin was investigated in two ways: by the use of growth retardants and analysis of *in vivo* hormone levels.

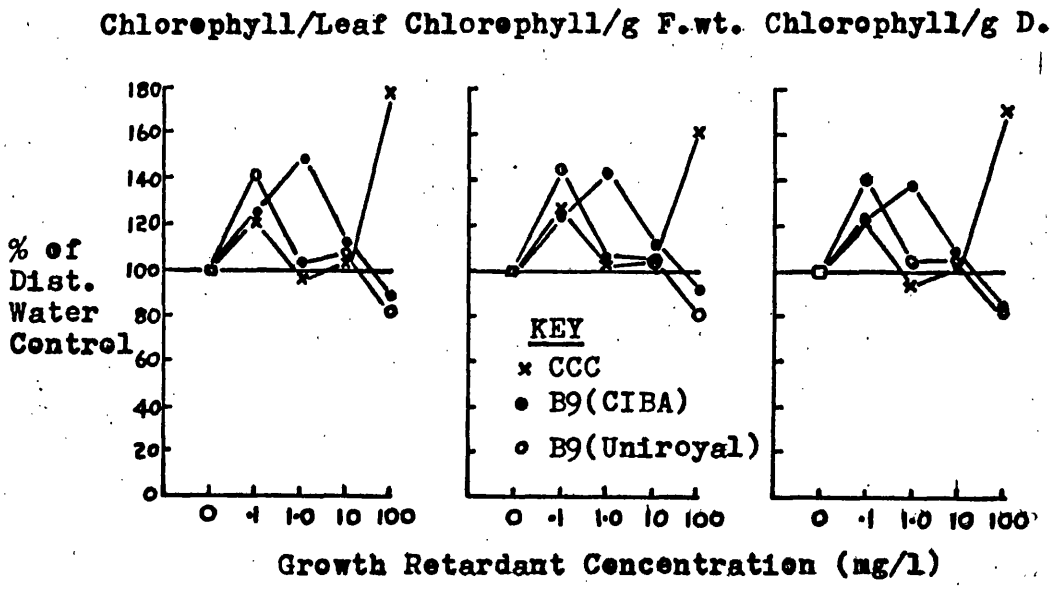
The growth retardant, CCC, inhibits the biosynthesis of gibberellins, (Kende, Ninneman and Lang, 1963; Harada and Lang, 1965; Zeevart, 1966). B9 does not inhibit gibberellin synthesis (Ninneman, Zeevart, Kende and Lang, 1964) and at present the mode of action of this compound is not known. The kinetics of its effects on plant growth are, however, similar to those of other growth retardants (Lang, 1970).

These retardants were applied at various concentrations to etiolated mung bean leaves which were subsequently illuminated. Two sources of B9 were used and both elicited similar responses. Chlorophyll synthesis was inhibited at a concentration of 100 mg/l of B9 and not at all by CCC (Figure 24). At 100 mg/l CCC actually promoted chlorophyll synthesis considerably as did B9 at lower concentrations. A concentration of 100 mg/l B9 was subsequently tested in the presence of exogenously supplied GA_3. In this experiment a promotion as a result of treatment with B9 alone was observed. The level of this was higher than that for the lowest

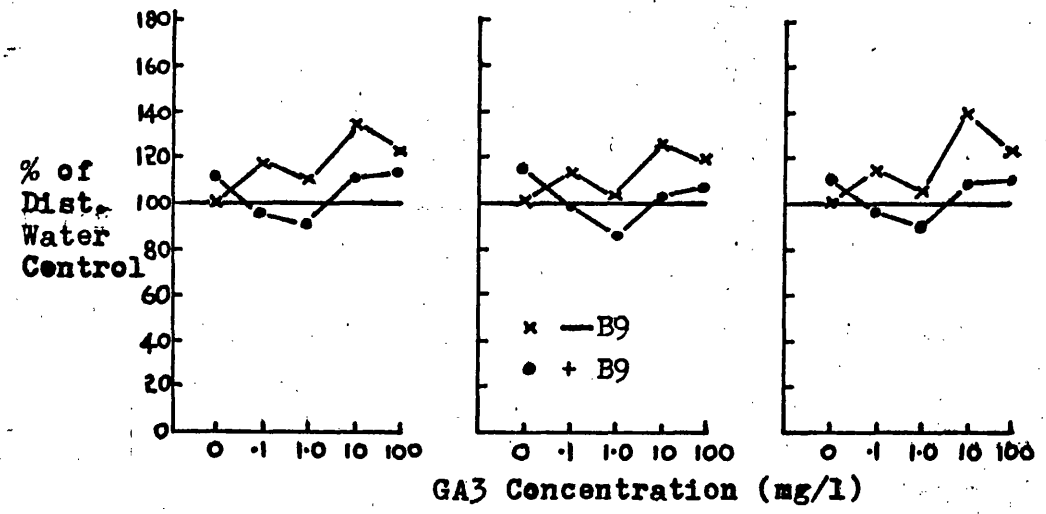
FIGURE 24

EFFECT OF GROWTH RETARDANTS ON CHLOROPHYLL CONTENT OF PRIMARY LEAVES

a) CCC, B9(CIBA), B9(Uniroyal)



b) B9 ± GA3



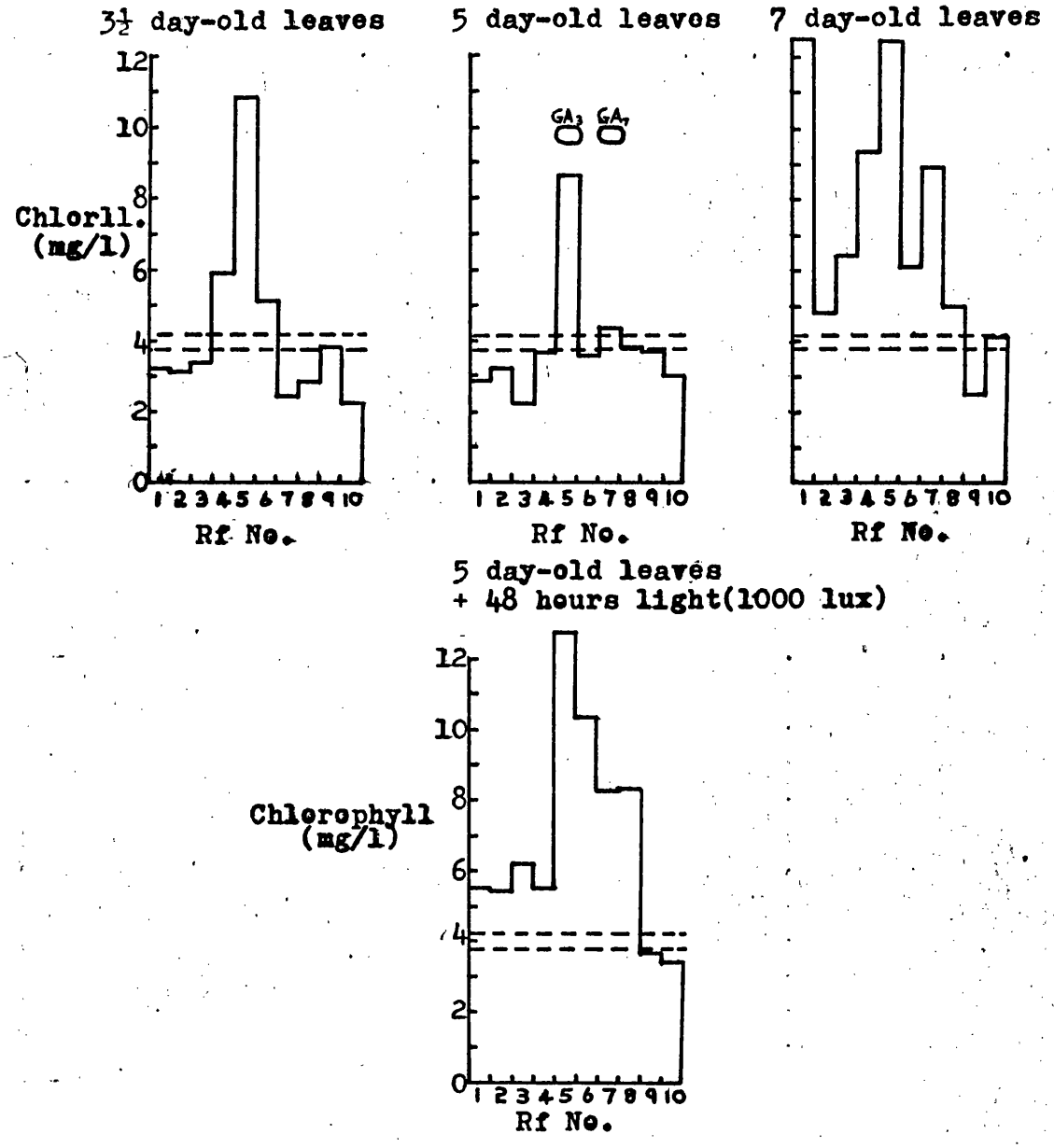
NOTE: 1. Illuminated at 1000 lux for 48 hours
2. Leaves from 5-day old plants

concentration of GA_3. It was evident however, that when added with GA_3, B9 interfered with GA_3 activity and the level of GA_3 promotion was reduced. It may be concluded that B9 either interacted specifically with GA_3 or interfered more readily with the activity of exogenously supplied gibberellin. These results do, however, indicate some effect due to the supplied GA_3, though they are not convincing evidence for a role played by endogenous gibberellins.

The levels ofmethanol soluble endogenous acidic gibberellin were assayed in three-and-a-half, five and seven day etiolated leaves and 5 day old leaves illuminated for 48 hours (Figure 25). In three-and-a-half day old leaves 35×10^{-6} µg equivalentsof GA_3 per leaf were measured but in five day old leaves the amount was less than 25% of this while after seven days etiolated growth the leaves contained 277×10^{-6} µg equivalent of GA_3 per leaf. Incubation of five day old leaves for 48 hours at 1000 lux resulted in an endogenous gibberellin level of 167×10^{-6} µg equivalent of GA_3 per leaf. Since no dark control was assayed, it was not possible to determine whether this increase was light stimulated or due to ageing of the leaf to seven days. There is considerable evidence for red light stimulation of gibberellin synthesis, (Reid, Clements and Carr, 1968; Kohler, 1966; Loveys and Wareing, 1971), and Wheeler (1960) has reported that the primary leaves of dark-grown *P. vulgaris* do not accumulate gibberellins but that light-grown leaves contain large quantities. Contrary to this Crozier and Audus (1968) observed less gibberellin in the leaves of light grown *Phaseoulus multiflorus* than in the leaves of the etiolated plant.

FIGURE 25

EFFECT OF AGE AND ILLUMINATION ON ENDOGENOUS GIBBERELLIN ACTIVITY* OF PRIMARY LEAVES



TOTAL GIBBERELLIN CHANGES

Treatment	GA/Leaf†	GA/g Dry wt.†
3½ days	35×10^{-6}	87×10^{-3}
5 "	8×10^{-6}	7×10^{-3}
7 "	277×10^{-6}	213×10^{-3}
5 " + Light	167×10^{-6}	210×10^{-3}

* as measured by Rumex leaf disc assay † ug eq. of GA3

On a dry weight basis the gibberellin changes were similarly related but the light-treated leaves possessed a much greater concentration.

When related to the growth curve, the highest gibberellin content occurred just prior to stem development and at the end of cotyledon life.

An inspection of the qualitative changes (Figure 25) shows that three and five day old leaves contained single peaks at Rf 0.5. By seven days this peak had increased considerably and others at Rfs I and 7 were also present. In the illuminated leaves, peaks at Rfs 5, 6, 7 and 8 were present. These could possibly be resolved into two peaks. There was no peak at Rf 1, which indicated that this was a result of attachment to the plant. The gibberellin activity at Rfs 5 and 7 corresponded to the Rf values for GA_3 and GA_7.

In relation to the results obtained from the effects of hormones on chlorophyll synthesis, these data show that the exogenously applied hormones were most active when the endogenous level was low. The internal hormone status of the leaf, therefore may determine the level of response of the leaf to exogenously added hormones.

4. Development of Carbon Dioxide Changes and the Effect of Hormones and Substrate

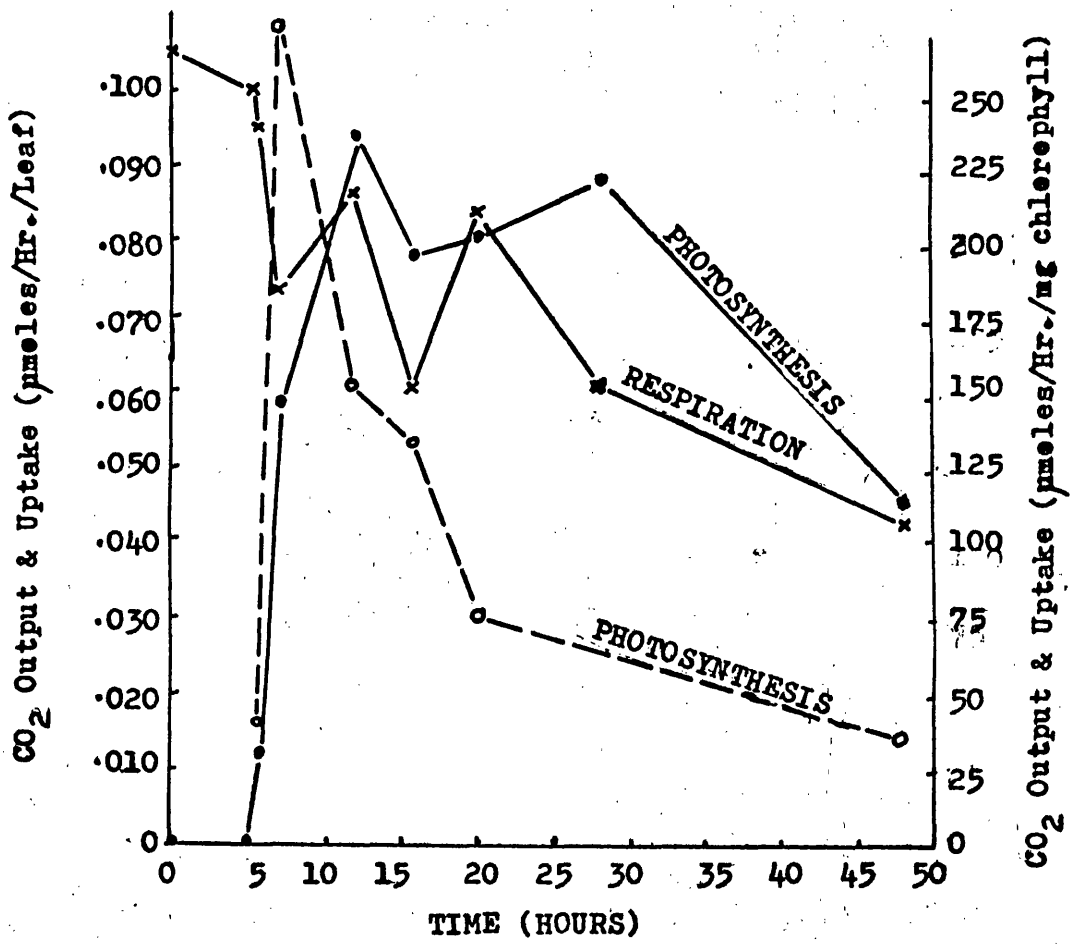
Carbon dioxide exchanges in greening leaves were examined because of the association of chlorophyll synthesis and photosynthetic development and the possible role of hormones in controlling photosynthetic and respiratory activity.

The respiratory changes have been plotted on a per leaf basis only, because the curves on fresh weight and dry weight bases were very similar. For the same reason, photosynthesis was plotted in this way. In addition, however, itsrelationship with the development of chlorophyll has also been shown by expressing it per unit of chlorophyll, (Figure 26).

The purpose of this investigation was to determine at what time photosynthesis began and to follow its subsequent development and that of respiration. After 5 hours illumination, no uptake of carbon dioxide was observed, but a further half an hour was sufficient to cause a detectable carbon dioxide influx. This rose rapidly to a maximum after about 12 hours illumination and then declined. On a chlorophyll basis a sharp maximum was reached after 7 hours illumination. The carbon dioxide uptake then dropped rapidly until 20 hours of illumination and subsequently decreased more slowly. In the early stages the increase in photosynthetic activity was much more rapid than the increase in the chlorophyll level, but later chlorophyll accumulation did not result in

FIGURE 26

TIME COURSE OF DEVELOPMENT OF PHOTOSYNTHESIS
AT 1000 LUX



KEY

x——x Respiration/leaf

●——● Photosynthesis/leaf

o——o Photosynthesis/mg Chlorophyll

NOTE: 5-day old leaves

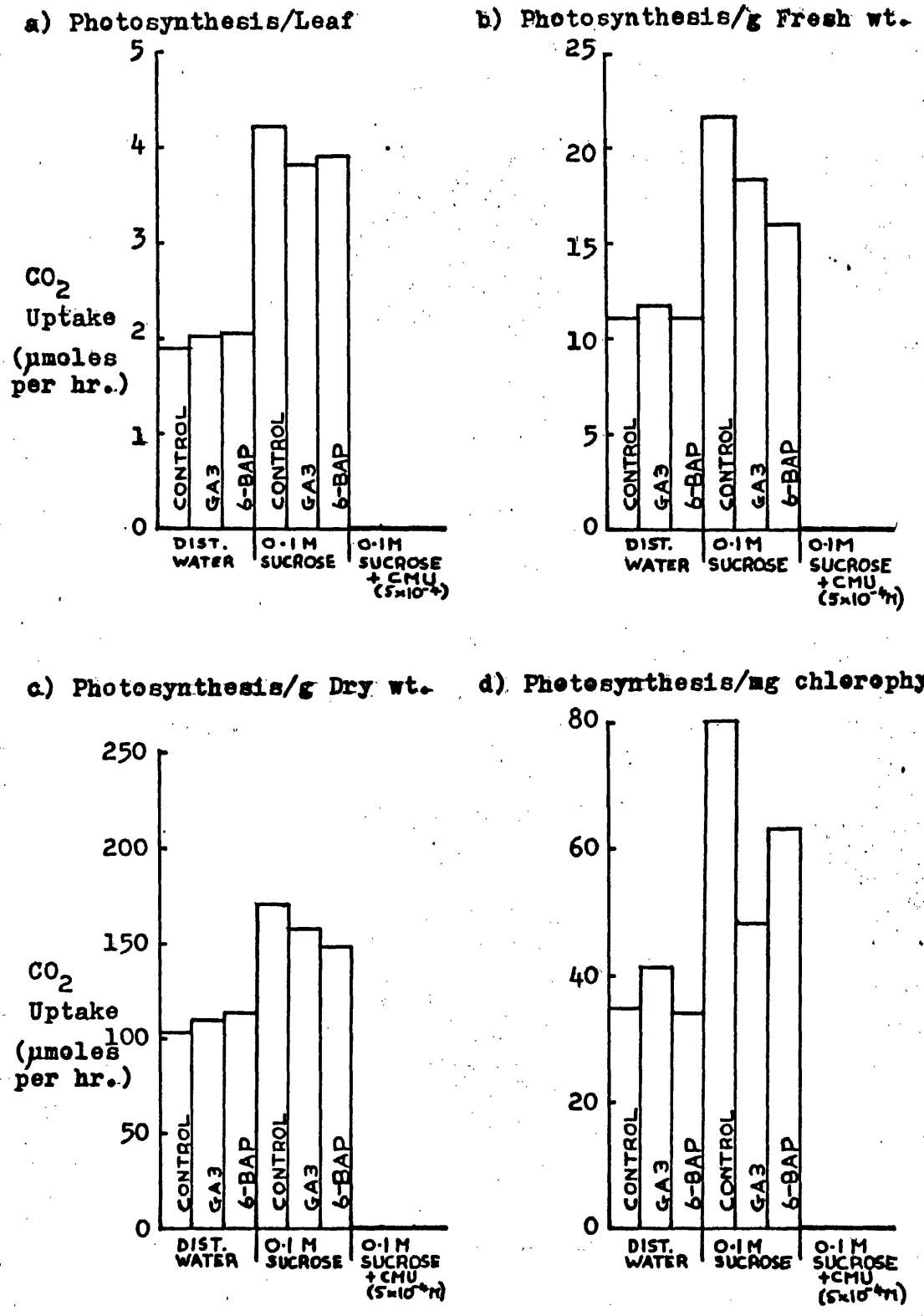
increased photosynthesis. The rate of respiration constantly dropped during the incubation; the decline was more rapid during the first 24 hours. The leaves did not compensate for respiration until after at least 12 hours of illumination. This correlates with the effect of CMU (Dodge *et al*, 1971) on chlorophyll synthesis, when inhibition began at some stage between 10 and 20 hours.

The development of photosynthesis per leaf was different from that observed by Bradbeer (1969) for *P. vulgaris*. He did not detect carbon dioxide uptake until after 15 hours illumination and the rate rose proportionally with time during the 48 hours light period. Apart from the species of plant used, the major differences in the experimental conditions were light intensity and the nature of the plant system. The *P. vulgaris* leaves were illuminated at 3000 - 4000 lux and were attached to the whole plant, but the mung bean leaves were incubated alone. The presence of the hypocotyl and root could act as a sink for substrate from the developing photosynthetic system and thus sustain an increase in photosynthetic rate (Humphries and French, 1969). This continued increase in photosynthetic rate was also observed in whole pea seedling (Dowdell and Dodge, 1971) and barley seedlings (Rhodes and Yemm, 1966). The present results would appear to be a feature of detachment from the plant.

Photosynthesis by the whole leaf was not significantly enhanced by either GA_3 or 6-BAP (Fig.27), although small increases were present. When expressed as per unit chlorophyll, only GA_3 showed a small increase. The addition of 0.1 M sucrose markedly

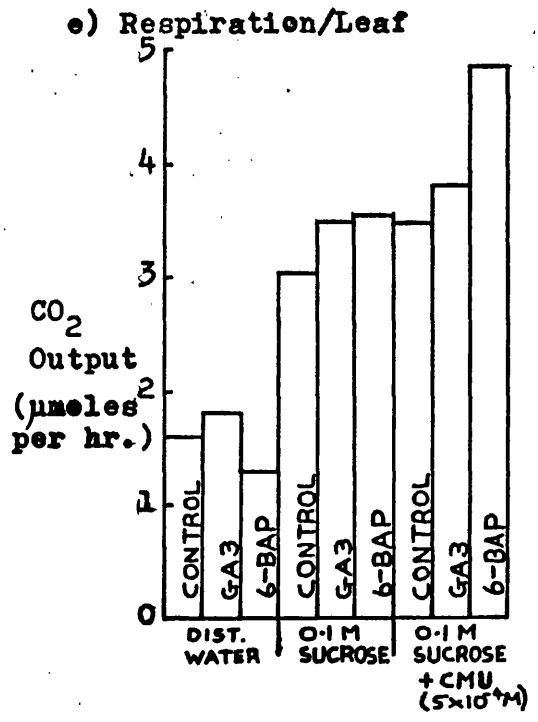
FIGURE 27

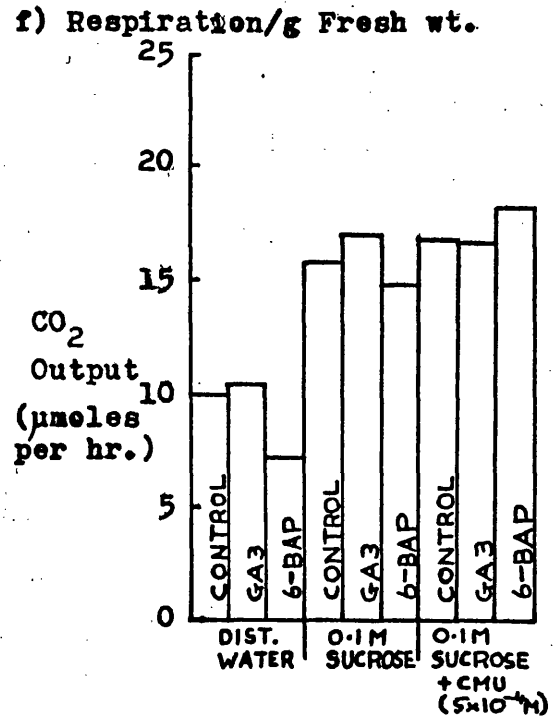
PHOTOSYNTHESIS AND RESPIRATION OF PRIMARY LEAVES : EFFECT OF GA3 AND 6-BAP ± SUCROSE AND SUCROSE/CMU

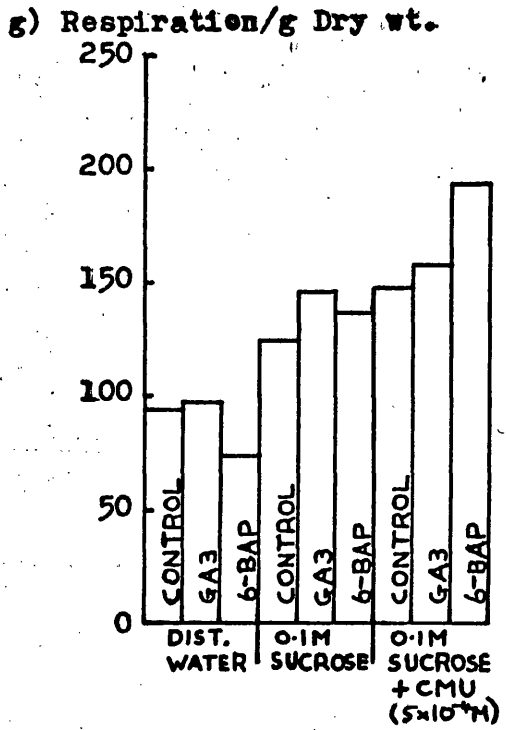


(continued on next page)

FIGURE 27 (Continued)







NOTE: 5-day old leaves illuminated for 48 hours

stimulated the photosynthetic rate on whichever basis it was expressed, but when GA_3 or 6-BAP was added to this, the photosynthetic rate was slightly reduced. Because of the hormone increases in fresh weight and chlorophyll, the reductions were more evident when expressed in these terms. This implied that the chlorophyll produced in response to hormone treatment was not contributing to photosynthesis. As might have been expected, there was no photosynthesis in leaves treated with CMU.

In the control system, GA_3 did not affect respiration but 6-BAP inhibited it. Sucrose promoted the rate of respiration and it was further enhanced by the presence of GA_3 or 6-BAP. This was also the case in the CMU/sucrose system, but it is worth noting that in this system 6-BAP was considerably more effective than GA_3.

Although the changes elicited by the hormones after 48 hours may be of significance in connection with their effects on chlorophyll synthesis, the changes at earlier stages may have been equally relevant to the situation at this stage.

5. The Effect of GA_3 and 6-BAP on Cell and Chloroplast Number

The chlorophyll content of the leaf may be increased either by an increase in the number of chloroplasts or in the amount of chlorophyll within each chloroplast and an increased number of chloroplasts may result from an increase per cell or an increased number of chloroplast-containing cells. The purpose of this part of the investigation was to determine if the various treatments affected any of these parameters. The results in Table 3 show that the number of chloroplasts in a mung bean leaf cell was not significantly altered by any of the treatments. Similarly the number of chloroplast-containing cells was fairly constant. These results indicate that the treatments affected the synthesis of chlorophyll of the developing chloroplasts by influencing the developmental processes rather than the replicative ones.

TABLE 3

EFFECT OF VARIOUS TREATMENTS ON CELL AND CHLOROPLAST NUMBER

Treatment	Cells/leaf	Chloroplast containing cells/leaf	Chloroplasts /cell
Control	2.3×10^7	1.4×10^7	17
GA3 (1mg/l)	2.4 "	1.7 "	19
6-BAP (5mg/l)	2.2 "	1.5 "	19
Sucrose (0.1M)	2.6 "	1.8 "	18
GA3 + Sucrose	1.9 "	1.4 "	17
6-BAP + Sucrose	2.2 "	1.5 "	18
Sucrose + CMU (5×10^{-4})	2.1 "	1.5 "	20
GA3 + Sucrose +CMU	2.4 "	1.8 "	18
6-BAP + Sucrose +CMU	2.4 "	1.6 "	19

DISCUSSION

DISCUSSION

I. Gibberellins and Chlorophyll Synthesis

Gibberellic acid elicited a concentration-dependent promotion of chlorophyll synthesis. The maximum response was similar in 3 to 7 day old leaves but in the young leaves inhibition at low GA_3 concentrations was observed. Many reports have indicated that the application of GA_3 gives rise to leaves of a paler green colour than those of untreated plants (Wittwer and Bukovac, 1958; Sestak and Ullman, 1960; Wolff and Haber, 1960; Wheeler and Humphries, 1963; Hamburg, 1964; Szalai, 1968, 1969; Grebenskii and Palanitsa, 1970). In mung bean leaves under the stated conditions this was not the case, since increased chlorophyll content was observed. All of the above reports were based on results from the treatment of whole plants. Wolff and Haber, (1960) investigated the effect of GA_3 on detached etiolated leaves and they observed no change in the rate of synthesis of chlorophyll as a result of GA_3 treatment. In these experiments 8 day old wheat leaves were used, and this may explain the difference in response. Beevers *et al* (1970) also observed no change in chlorophyll synthesis during the lag phase in 7 day old detached etiolated wheat leaves. The leaves were treated during a six hour pre-incubation period, washed and then illuminated on distilled water. This approach would have been valuable in the mung bean system for determing the stage during illumination when the leaves were most

responsive and for what period the hormone remained active after the exogenous source had been removed.

It has been suggested that the paler appearance of whole plants treated with GA_3 is not due to decreased chlorophyll synthesis but is an artefact caused by increased cell expansion (Wolff and Haber, 1960; Bishop and Whittingham, 1961; Wheeler and Humphries, 1963). Wheeler and Humphries (1963) found that treated potato leaves contained more chlorophyll/leaf than untreated ones but less chlorophyll/gram fresh weight. In peas (Bishop and Whittingham, 1961) chlorophyll/leaf and chlorophyll/gram fresh weight were the same in treated and untreated plants while in wheat, Wolff and Haber (1960) observed that total chlorophyll content was unchanged in 6 day old plants but was less in older plants treated with GA_3. It has also been proposed that the GA_3-induced chlorosis results from reduced chlorophyll synthesis and/or enhanced chlorophyll destruction. Sestak and Ullman (1960) recorded that chlorophyll/gram dry weight was greatly decreased by GA_3 during the greening of 7 day old wheat and maize seedlings. They concluded that this reduction was due to the preferential utilization of substrate in GA_3-promoted syntheses. A decrease may have been detected because the treated plants were dark-grown for 7 days in nutrient solution containing GA_3 and not treated just before illumination. The dry weight values for the leaf samples showed that GA_3 reduced the dry weight of the leaves during this dark development. Thus the effect on chlorophyll synthesis was complicated by changes prior to illumination. These results were, however, supported by those of Artomonov (1966) who found decreased chlorophyll/gram dry weight in

sugar-beet sprayed with GA_3 in the field and 10 day old etiolated corn seedlings treated in the laboratory. He concluded that GA_3 both decreased chlorophyll synthesis and enhanced chlorophyll destruction. The same conclusion was reached by Grebenskii and Palanitsa (1970) for maize, pea, *Amaranthus* and *Codetia*. Szalai (1968) reported that the leaves of *P. vulgaris* became chlorotic when light-grown plants developed in the presence of GA_3. The decrease in chlorophyll level was proportional to increasing gibberellin concentration. The same relationship was observed when GA_3 was applied to *Hordeum vulgare* seedlings (Szalai, 1969). In both papers chlorophyll was based on fresh weight and no "whole leaf" data were reported. Also, the plants were treated with GA_3 for three days prior to illumination. These experimental differences may well account for the disagreement with the results in *P. aureus*. Using only the etiolated leaves of *P. vulgaris* Wolff and Price (1960) and Sisler and Klein (1963) detected no responses as a result of GA_3 treatment.

The results with *P. aureus* support the 'dilution' theory explained above, and show that GA_3 can promote the chlorophyll level. The reduction of chlorophyll content at some concentrations and at certain leaf ages suggests that decreased synthesis or enhanced destruction may occur. Karabanov (1968) reported that in black-currant leaves under field conditions, both inhibition and promotion occurred depending on the availability of mineral nutrients. Gibberellic acid alone was slightly inhibitory, but in the presence of nitrogen, phosphorus and potassium it increased the chlorophyll

content. In corn leaves (Artomonov, 1966) the addition of riboflavin not only reversed the observed GA_3 inhibition but resulted in an enhanced chlorophyll level when compared with riboflavin alone. The conditions of nutrient supply, therefore, appear to affect the response of the chlorophyll level to GA_3. The results in Figure 21 show that the addition of various carbohydrates enhanced the effect of GA_3 but dependended on the concentration of the substrate.

GA_3 was most effective on a percentage basis at the beginning of photosynthesis. This and the absence of any effect in the presence of CMU also suggests that GA_3 activity in this system was limited by substrate availability. Another variable factor between many of the reports was the use of either etiolated or green leaf material. It was not possible, however, to separate the promotive and inhibitory effects of GA_3 on this basis.

2. Cytokinins and Chlorophyll Synthesis

6-Benzylaminopurine inhibited and promoted the level of chlorophyll in mung bean leaves aged 3½ and 5 days. The direction of the response depended on the concentration supplied. In seven day old leaves, 6-BAP inhibited at all concentrations. Sugiura (1963) reported that kinetin at 10^{-5}M (Ω 2mg/1) promoted chlorophyll synthesis in 6 day old etiolated *P. vulgaris* leaves after 3 hours illumination in the presence of the cotyledons. There was, however no effect in their absence. This early promotion was consistent

with the one observed in five day old etiolated mung bean leaves and agrees with that observed in etiolated cucumber cotyledons (Fletcher and McCullagh, 1971) and etiolated barley leaves (Beevers *et al*, 1970). Fletcher and McCullagh reported that the age of the cotyledons and length of exposure to light were critical factors in working with low concentrations of cytokinin. Narain and Laloraya (1970) found that kinetin inhibited chlorophyll synthesis in cucumber cotyledons after 48 hours illumination, while Hardy, Castlefranco and Rebeiz (1971) observed no effect after 3 hours illumination. In a previous paper Banerjii and Laloraya (1967) had demonstrated that in pumpkin cotyledons kinetin stimulated chlorophyll synthesis. Similarly Penner and Wiley (1972) reported that 6-BAP promoted chlorophyll synthesis in cucumber and squash cotyledons after only 8 hours illumination. It would seem therefore that the effect varies with the plant species and the period of incubation, as it does in mung bean leaves. The observed stimulation was largely a result of increased protochlorophyll synthesis rather than an effect on its photoreduction to chlorophyll. Shlyk and Averina (1969) have also shown that kinetin stimulated protochlorophyllide synthesis in green barley leaves incubated in the dark. Stobart, Shewry and Thomas (1972) observed that kinetin at 0.16 mM (Ω 36 mg/l) promoted chlorophyll synthesis in etiolated barley leaves illuminated for 16 hours. The effect was absent in leaves up to eight days old and became evident as the leaves aged from eight to twenty days. This observations was the converse of the response of mung bean leaves. In cultured branch apices of the red algae *Hypnea musciformis* kinetin stimulated chlorophyll *a* and phycoerythrin synthesis

(Jennings, Broughton and McComb, 1972) and the role of cytokinins in retarding chlorophyll degradation in senescing leaves is well documented (Richmond and Lang, 1957; Mothes, 1959; Osborne, 1962 and 1967).

Investigations using tissue culture have shown that kinetin can promote chlorophyll concentration in olive callus growth, (Lavee and Messer, 1969). In non-green callus tissue from *Nicotiona tabacum* L. the presence of kinetin and sucrose were essential for the induction of chlorophyll synthesis (Kaul and Sabkarwal, 1971). In green callus, however, kinetin inhibited chlorophyll development. Kinetin and sucrose interacted in promoting chlorophyll synthesis in the non-green callus. At low sucrose concentrations (0.05 and 0.1%) chlorophyll synthesis increased with increasing kinetin concentration up to 6 mg/l and decreased at 8 mg/l. At sucrose concentrations up to 6%,inhibitions by both low and high kinetin concentrations were observed with an optimum promotion at 4 mg/l. These results were obtained one week after the induction of greening. After a four week interval the inhibitions were overcome. Inhibitions by low concentrations of kinetin were also found in mung bean leaves (Table 1 and Figure 18) and the response was altered by the presence of sucrose. Sugiura (1963) tried to replace the function of the cotyledon in the effect of kinetin on chlorophyll synthesis, with 0.2 M sucrose. After 3 hours illumination no effect was evident. The results from tobacco callus showed that it was necessary to test a number of concentrations of each substance to obtain a comprehensive assay. In mung bean leaves the results indicated that sucrose suppressed the effect of 6-BAP. This and the data of Suguira (1963)

suggest that 6-BAP activity was not specifically dependent on carbohydrate supply. Its promotive effect in the presence of the cotyledon may have been due to increased translocation from the cotyledon to the leaf. With the exception of the data of Narain and Laloraya (1970) results from the application of 6-BAP or kinetin to etiolated cotyledons indicated that cytokinins were very effective in this system. Penner and Wiley (1972) concluded that this may be a result of increased availability of cotyledon reserves as a result of 6-BAP treatment. Application of cytokinins to isolated etiolated leaves has in many instances elicited no response (Wolff and Price, 1960; Sisler and Klein, 1963; Hardy *et al*, 1971). Positive effects have been observed only in mung bean and wheat leaves (Beevers *et al*, 1970). The percentage increase of chlorophyll in leaves treated with 6-BAP was low in comparison with that in treated cotyledons. This is consistent with the conclusion of Penner and Wiley (1972) since the leaves contained less reserve than the cotyledons, but indicated that cytokinins are capable of other effects on chlorophyll synthesis.

Both 6-BAP and kinetin were applied to mung bean leaves and the responses to the two cytokinins were very different at the tested concentrations. Kinetin inhibited and 6-BAP promoted chlorophyll accumulation. On the addition of sucrose the kinetin induced inhibition was reversed and became higher than the control. The effect of 6-BAP was either reduced or unaltered depending on the sucrose concentration. It may be concluded that under the conditions of the experiment the two cytokinins, kinetin and 6-BAP, exerted

different responses. Fletcher and McCullagh (1971) observed that zeatin, kinetin and 6-BAP were all equally effective in promoting chlorophyll synthesis in cucumber cotyledons. Differences in the response to gibberellin type were also evident in mung bean leaves, although none of them inhibited chlorophyll synthesis at the concentrations supplied. The addition of sucrose (0.1 and 0.2 M) to the medium enhanced the gibberellin effect which sucrose at 0.05 and 0.01 M caused GA_7 to become inhibitory. The differences between the various hormones may be due to different concentration optima for each of them.

3. Interaction of GA_3 and 6-BAP

When GA_3 and 6-BAP were applied in combination a synergistic response was obtained. Its presence was dependent on the age of the leaves and the concentrations of the two hormones and was related to the level of endogenous gibberellin activity assayed in the leaves prior to treatment. The pattern of the relationship of the two hormones indicated that the response of chlorophyll synthesis to a combination of GA_3 and 6-BAP was determined to a greater extent by the cytokinin concentration. The maximum response always occurred at 5 mg/l. The response to GA_3 was a much broader one and lower concentrations of this hormone were more effective in promoting chlorophyll synthesis at higher 6-BAP concentrations. At the lower 6-BAP concentrations, the addition of GA_3 enhanced the 6-BAP inhibition of the chlorophyll level. It may be concluded that

6-BAP enhanced the effectiveness of GA_3. Both Bejerano and Lips (1970) reported a similar inter-relationship for nitrate reductase activity in summer-grown tobacco plants. In the winter-grown plants the relationship altered such that as the concentration of kinetin was increased the greater was the concentration of GA_3 required to obtain maximum activity. It was suggested that winter-grown tobacco contained higher endogenous levels of cytokinin, therefore addition of kinetin resulted in supra-optimal concentrations. The results obtained from mung bean leaves cannot be explained on the basis of the endogenous gibberellin level since 6-BAP should have promoted in the leaves with the highest content i.e. seven days. This would suggest that the action of 6-BAP in a combination of hormones was specifically related to exogenous gibberellin. The overall response, however, does as has already been stated, show an inverse relationship with the endogenous gibberellin level.

In isolated pumpkin cotyledons GA_3 (1 mg/l) and 6-BAP (10 mg/l) given in combination promoted expansion to a greater extent than the sum of the individual hormone effects. These concentrations are very similar to those used in mung bean leaves. The promotion of chlorophyll synthesis by these hormones did not result from an effect on leaf expansion, since the pattern of response for fresh weight data was different from that for chlorophyll synthesis. In leaves of *P. vulgaris*, GA_3 accelerated leaf expansion with the final size of the leaf unaltered (Humphries, 1958). Kinetin depressed both of these parameters and the two hormones together followed the GA_3 curve. An additive response to GA_3 and kinetin was observed when the two hormones were applied to etiolated bean leaf discs for

24 hours in the light (Humphries and Wheeler, 1960). In mung beans the hormones increased the fresh weight of the isolated leaves and the effect was greater as the hormone concentration increased. The interaction showed synergism, but this occurred at the combination of the highest hormone concentration and not at the concentrations which were optimal for chlorophyll promotion. It appears unlikely therefore that the hormones improved the leaf chlorophyll content by increasing leaf expansion and providing a greater light-receptive area.

A change in the batch of beans used for experimentation resulted in an absence of response to the most effective combination of GA_3 and 6-BAP as assayed in the original material. In this second batch leaf chlorophyll was increased by both hormones when added individually but their interaction had altered. Originally, exogenous gibberellin promoted most at the 6-BAP concentration which gave maximum promotion, but in the second batch it was antagonistic. This may have been due to a higher endogenous level of gibberellin or a changed substrate status. The addition of sucrose to the medium (Table 2) did not replace the effect of GA_3 but it did upset the balance of the two hormones with the result that the high concentrations gave greatest promotion. The substrate status of different batches of seeds could therefore have altered their response to hormone concentration. The level of endogenous hormone may have been changed by this and consequently its relationship with the applied hormones may not have been causal. Conversely it is possible that the hormone level may control substrate availability.

The age effect (Figure 23) was possibly determined by both of these factors. Sisler and Klein (1963) observed that the older leaves of *P. vulgaris* required exogenous sucrose to obtain a reasonable rate of chlorophyll synthesis. It may be concluded therefore, that as etiolated leaves age they lose substrate. The five day old mung bean leaves probably represented an intermediate state where there were considerable reserves but a low endogenous hormone level. Treating the leaves at this stage with exogenous hormone therefore resulted in the greatest interaction of endogenous reserves and exogenous hormone. A reduction in substrate, brought about by the inhibition of photosynthesis by CMU in the presence of sucrose, abolished the effects of the combinations of high concentration of 6-BAP and GA_3 observed with sucrose alone. There were signs of synergism between the two hormones when applied at low concentrations. Jones and Kauffman (1971) found that kinetin inhibited gibberellin-promoted growth of *Avena* internodes and the inhibition was much greater in the absence of sucrose. Kinetin appeared to direct substrates for use in other synthetic processes. The interaction of the two hormones was of a different nature in mung bean leaves although the addition of sucrose did result in an additive interaction at concentrations which previously had resulted in mutual interference.

The roles of GA_3 and 6-BAP in relation to one another present a complex problem, particularly as batches of seed differ from one another. If this is to be resolved it will be necessary to produce seed of a consistent and known nature. The most profitable techniques would seem to be:

a) the analysis of endogenous hormone and substrate status prior to use;

b) the use of material with genetically reduced hormone level; and

c) the use of material with chemically reduced hormone levels.

The last of these requires the use of inhibitors of hormone synthesis and this can result in more problems than it solves since the inhibitors can cause changes in growth patterns of plants (Stoddart, 1965; Felippe and Dale, 1968). An aspect of the interaction which may prove important with regard to the mode of action of the hormones is their sequential relationship. Treatment with kinetin prior to GA_3 increased the response of aleurone tissue α-amylose production (Eastwood, Taverner and Laidman, 1969) and of lateral shoot growth in tomato (Catalano and Hill, 1969).

4. Hormones and Substrate Supply

The concentration response of GA_3 and 6-BAP alone (Figure 18) corresponded with the general conclusion from the interaction data that the GA_3 response was governed by the 6-BAP system. Under each of the substrate regimes tested GA_3 showed a similar concentration curve, except in the absence of substrate, while the 6-BAP response varied. This suggested that GA_3 always acted in the same way but that 6-BAP was effective through two active sites. Although the pattern of response to 6-BAP alone was altered by different substrate

systems the magnitude of promotion was not markedly increased (Figure 17). The magnitude of GA_3 promotion, was, however, increased by substrate addition. In the system where photosynthesis was inhibited 6-BAP was also inhibitory. This could be reversed only by adding a high concentration (0.2 M) of sucrose and is evidence that 6-BAP promotion of chlorophyll synthesis was associated with photosynthesis. The time course of the effect of 6-BAP in the control system also showed a strong association with the onset of photosynthesis. The latter began after 5½ hours illumination and the largest 6-BAP promotion occurred after 7 hours. Sucrose abolished this initial increase but when photosynthesis was inhibited it was once again evident. This was the converse of the situation at 48 hours and thus indicated a temporal differentiation of the hormone effect. Gibberellic acid also greatly increased the level of chlorophyll immediately after the onset of photosynthesis in the control system, but this was not present in any of the other systems and is consistent with the suggestion previously made that its action is dependent on the provision of substrate. The magnitude of the early effects correlated with the very low hormone level at this stage. After 48 hours, the effect of the exogenous hormone decreased and the level of endogenous gibberellin increased. This latter change may have contributed to the decrease of hormone effectiveness.

GA_3 and kinetin have been shown to increase the photosynthetic rates of maize, bean and clover (Wareing, Khalifa and Treharne, 1968; Treharne and Stoddart, 1968), and these were

correlated with increased RuDP carboxylase activity in maize and clover leaves. Gibberellic acid similarly affected enzyme activity in pea leaves and stem internodes (Broughton, Hellmuth and Yeung, 1970) and 6-BAP increased carbon dioxide uptake in bean plants (Adedipe, Hunt and Fletcher, 1971). Feierabend (1969) reported that kinetin restored the rate of photosynthetic enzyme formation in rootless developing seedlings to that of intact seedlings. In this system gibberellic acid was ineffective. Gibberellic acid and 6-BAP very slightly increased leaf photosynthesis in mung bean leaves illuminated for 48 hours, but these effects were not significantly different from the control. On a chlorophyll basis, however, GA_3 was more effective. The situation at 48 hours may not reflect the true effect of these hormones, because the relevant changes may occur prior to this stage. Although the addition of sucrose enhanced the photosynthetic rate, GA_3 and 6-BAP were inhibitory in this system. These data do not exclude a role for photosynthesis in the mode of action of the hormones during mung bean leaf development.

Broughton and McComb (1971) advanced the hypothesis that the overall effect of GA_3 was to provide more substrate for general cell metabolism in elongating pea internodes, by stimulating the activity of enzymes involved in carbohydrate metabolism. Similar GA_3-increased enzyme activity in the leaf sheath of dwarf maize seedlings was observed by Katsumi and Fukahara (1969) and in tobacco leaves (Lee, 1969). GA_3 is well known to increase α-amylase activity in barley endosperm (Paleg, 1960). Broughton *et al* (1970) were unable to detect increased amylolytic activity in leaves of the treated peas, however, in their experiments the whole plant was

used and the hormone was injected into the internode. In mung bean leaves the effect of GA_3 on chlorophyll was increased by the addition of several different carbohydrates. The role of GA_3 in increasing substrate provision for metabolic processes seems to fit this data, but it is possible that the effect of GA_3 is merely dependent on the extra substrate. It has recently been reported (Delmer, 1972) that GA_3 promoted sucrose synthetase activity in the direction of sucrose breakdown when added to a preparation of the enzyme from *P. aureus*. Additional substrate did not enhance 6-BAP promoted chlorophyll synthesis. The data for hormone effects on respiration showed that GA_3 and 6-BAP increased this, thus indicating that both hormones stimulated the utilization of substrate. One may conclude therefore that GA_3 directed the substrate more specifically towards chlorophyll synthesis but that 6-BAP stimulated general use of the substrate for cell and leaf development. This was reflected in the larger dry weights generally observed for 6-BAP/sucrose treated leaves. It has previously been reported that kinetin increased starch breakdown in Chinese cabbage (Berridge and Ralph, 1971) and wheat coleoptiles (Boothby and Wright, 1962).

5. Chloroplast Replication

There are various functions which may result in an increase in chlorophyll level. These are increases in the amount of chlorophyll in each chloroplast, the number of chloroplasts in a cell or the number of cells containing chloroplasts. The last two are consequences of replication, and therefore, nucleic acid metabolism while the first results from changes in metabolic processes. Chloroplasts contain DNA and are capable of replication (see Introduction - Section 10) and cytokinins have been shown capable of increasing the number of chloroplasts in tobacco leaves (Boasson and Laetsch, 1969) and spinach (Possingham and Smith, 1972) and of controlling DNA replication in *Fumaria* chloroplasts (Giles, 1971).

In GA_3- and 6-BAP-treated mung bean leaves no difference in the number of chloroplasts per cell was observed, whether the system was supplemented with sucrose or not. Humphries and Wheeler (1960) reported that in the light, GA_3 and kinetin did not increase cell number in leaf discs of *P. vulgaris*. No significant increases were noted in the treated mung bean leaves. It may, therefore be concluded that the hormones did not exert their effects through DNA synthesis.

6. Protein Synthesis, GA_3 and 6-BAP

Chlorophyll synthesis is controlled by the formation of both cytoplasmic and chloroplastic proteins (see Introduction). Both GA_3 (Varner, 1964). Fletcher and Osborne, 1965; Jacobsen and Varner, 1967; Jones and Stoddart, 1970) and 6-BAP (Parthier and Wollgiehn, 1961; Sugiura, Umemura and Oota, 1962; Osborne, 1962; Kuraishi, 1968; Richmond, Sachs and Osborne, 1971) have been associated with the promotion of protein synthesis. More specifically, chloroplast protein synthesis in tobacco chloroplasts extracted from 6-BAP-treated leaves was greater than in those from control leaves (Romanko, Khein and Kulaeva, 1968; Richmond *et al*, 1971). In contrast, the increased activity of photosynthetic enzymes due to hormone treatment (Treharne and Stoddart, 1968) was not paralleled by ribosomal RNA synthesis which is normally associated with *de novo* protein synthesis (Treharne, Stoddart, Pughe, Paranjothy and Wareing, 1970). In isolated pumpkin cotyledons kinetin was able to partially overcome the inhibition of protochlorophyll synthesis induced by chloramphenicol (Banerjii and Laloraya, 1967) which suggested that its action may be at a similar level to that of chloramphenicol (see Introduction - Section 9) or that it bypassed the site of inhibition. Chloramphenicol was also ineffective in preventing kinetin-stimulated betacyanin synthesis in *Amaranthus caudatis* (Koehler, 1972) but cyclohexamide and actinomycin D both prevented the stimulation. Koehler (1972) concluded that kinetin may act at the level of gene de-repression but it could be equally true that its influence was exerted at the ribosomal level.

In red algae, however, chloramphenicol completely inhibited any kinetin effect on pigment synthesis (Jennings, Broughton and McComb, 1972). This was indicative of the requirement for plastid protein synthesis to sustain kinetin activity. The inhibitions produced by chloramphenicol and cyclohexamide on the formation of RuDP carboxylase in rye seedlings were not overcome by kinetin (Feierabend, 1970) although kinetin alone stimulated the rate of enzyme formation (Feierabend, 1969). It was stated that under appropriate conditions all the enzymes studied responded to variation in the cytokinin level thus indicating an unspecific role in this respect. This may be compatibile with a general effect of cytokinin on gene activation or alternatively on increasing the demand for substrates from these enzymes.

The activity of alanine aminotransferase, which may be associated directly with chlorophyll synthesis (see Introduction - Section 6), is altered by hormone application. Its activity in the whole plant was increased by CCC and decreased by GA_3 (Hedley and Stoddart, 1971b). In leaf segments both kinetin and CCC retarded the decline in activity but GA_3 was without effect. Kinetin also retarded the loss of chlorophyll but neither GA_3 nor CCC was effective in this direction. These results are compatible with the stimulatory effects of 6-BAP and CCC in mung bean leaves, but not with the effects of GA_3.

7. Membrane Permeability

Recent evidence (Fondeville, Borthwick and Hendricks, 1966; Tanada, 1968) implicated membrane permeability as a primary site of action for phytochrome and the subject has been discussed by Hendricks and Borthwick (1967), Black (1968) and Smith (1970). The similar action of phytochrome and cytokinins in various systems (Miller, 1956; Hillman, 1957; Powell and Griffiths, 1960) and the red light stimulated production of gibberellins (Kohler, 1966; Reid, Clements and Carr, 1968; Loveys and Wareing, 1971) provide indirect evidence for associating hormone activity with this system. Some direct evidence has been obtained from isolated leaf protoplasts (Power and Cocking, 1970). The chloroplast envelope is a primary barrier between the cytoplasm and chloroplast and provides a suitable target. Richmond, Sachs and Osborne (1971) have suggested that kinetin may exert a primary effect on the hydration and permeability of chloroplast membranes. The permeability of the chloroplast membrane is of great importance during the development of the chloroplast. Prior to synthesis, it restricts the substrates which may pass into the developing etioplast and later the photosynthetic substrates which pass out, while throughout the whole of the life of the chloroplast it may regulate nuclear and cytoplasmic control.

8. Characteristics of GA_3 and 6-BAP Activity in Mung Bean Leaves

The effects of gibberellins and cytokinins appeared to be very similar in general terms. The data reported above suggest that the effects of GA_3 and 6-BAP on mung bean leaf chlorophyll synthesis have different characteristics. The results obtained when the hormones were applied alone and in combination indicated that 6-BAP activated two different sites and GA_3 one site in five day old leaves. The characteristics of these activities are summarised in Table 4. The site operated by low concentrations of 6-BAP inhibited chlorophyll synthesis under the conditions of the control system and the sites operated by high concentrations of both hormones stimulated. The effect of the 6-BAP-inhibited site was reversed by all treatments except low concentrations of GA_3. At high concentration of 6-BAP, GA_3 at all concentrations enhanced the effect. It was reversed by the addition of CMU but substrate was ineffective in the control or CMU system. The responses to substrate and CMU suggest that the low concentration 6-BAP site diverted general cell substrate away from chlorophyll synthesis, but that the high concentration 6-BAP site was dependent on photosynthetically produced substrate. The reversal of 6-BAP inhibition by the addition of CMU is inconsistent with this suggestion. This result was, however, obtained from only one experiment while the others were drawn from several. The close association of the high concentration 6-BAP site and photosynthesis raises the possibility that this site is located with the chloroplast. The low concentration

TABLE 4

CHARACTERISTICS OF HORMONE-ACTIVATED SITES IN 5 DAY-OLD GREENING MUNG BEAN LEAVES

Site	Control	+GA3		+6-BAP		+CMU	+Substrate	
		High	Low	High	Low		+CMU	-CMU
6-BAP* Low Conc.	-	R	E	R	/	R	R	R
6-BAP** High Conc.	+	E	E	/	/	R	R	O
GA3** High Conc.	+	/	/	E	R	R	E	E

KEY

O	No effect
+	Promotion
-	Inhibition
E	Enhancement
R	Reversal
/	No result

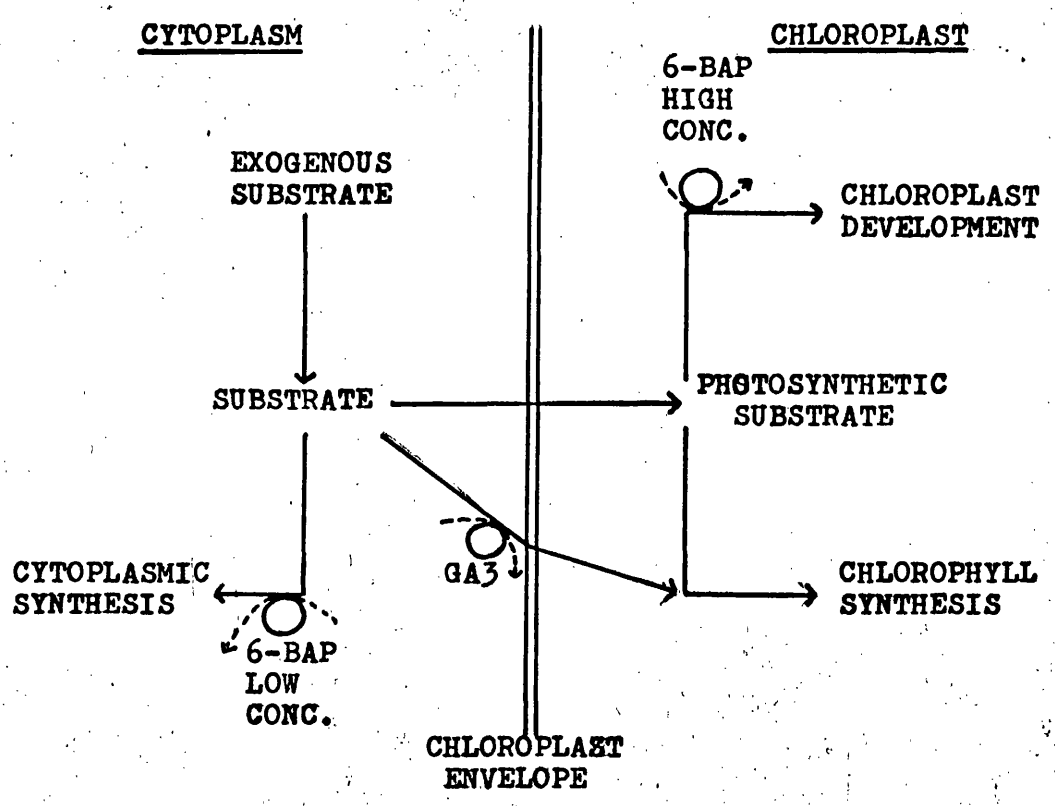
* 0.01-0.001 mg/l
** 1.0-10 mg/l

Note. Control column shows effect of hormone on chlorophyll synthesis : other columns show effect of respective additive on control result .

6-BAP site may be cytoplasmic. This is also supported on the basis that high concentrations may penetrate further than low concentrations. The GA_3-operated site was rendered ineffective in the absence of substrate (+ CMU) but was very effective when supplied with substrate whether photosynthesis was operating or not. This suggested that GA_3 enhanced breakdown and utilization of exogenous and possibly endogenous substrate. The tendency for high concentrations of GA_3 to reverse 6-BAP inhibition of chlorophyll synthesis may have been achieved in this way. The inability of exogenous substrate to alter the high concentration when photosynthesis was inhibited, indicated however, that GA_3 may not have behaved in this manner during the synergistic promotion. It has already been suggested that 6-BAP responded to photosynthetically produced substrate. Gibberellic acid could have specifically interacted with 6-BAP by increasing the supply and utilisation of this. The effects of GA_3 and 6-BAP on chlorophyll synthesis and their interaction with substrate, are represented diagrammatically in Figure 28. The interaction of the leaf hormone status and the changing nutrient status may explain the age response to the added hormones. In the youngest leaves both the hormone and nutrient level was high, but because the latter was much greater on a proportional basis exogenous hormones exerted some influence. In five day old leaves the hormone level was considerably reduced, but nutrient status was still fairly high thus the added hormones, particularly GA_3, increased the level of substrate utilization. After seven days growth, the leaf hormone level had increased and the nutrient level decreased resulting in reduced effectiveness of the endogenous hormones.

FIGURE 28

INVOLVEMENT OF GA3 AND 6-BAP IN CHLOROPHYLL SYNTHESIS AND THEIR INTERACTION WITH SUBSTRATE



KEY

○ SITE OF HORMONE ACTION

⟶ SUBSTRATE FLOW

- - - - -⟶ DIRECTION OF HORMONE ACTION

The interaction of GA_3 and 6-BAP is further complicated by the finding that cytokinins can increase or maintain endogenous gibberellin levels (Sebanek, 1966; Karanov and Vassilev, 1969; Chin and Beevers, 1970). The different natures of the gibberellin and cytokinin activities in the mung bean system make this an unlikely mode of action for cytokinin.

9. Endogenous Gibberellins, CCC and B9 Activity

Newly synthesized leaf gibberellin seemed to play little part in chlorophyll production as suggested by the failure of CCC to inhibit the chlorophyll level. At 100 mg/l CCC, chlorophyll synthesis was promoted. This has been observed previously in tobacco (Humphries, 1963), ryegrass (Stoddart, 1965) and French bean (Humphries, 1968). Inhibition of chlorophyll synthesis by CCC has been observed primarily in cotyledons (Negbi and Rushkin, 1966; Knypl, 1969; Knypl and Chylinska, 1972) but has also been reported in etiolated barley leaves (Shewry, Pinfield and Stobart, 1971; Berry and Smith, 1970). Some of the reports indicated that the inhibition of protein synthesis was a possible mode of action. Berry and Smith (1970) concluded that this may be the effect of CCC concentrations greater than 10^{-3} M (Ω 166 mg/l) which may explain the effects observed in cotyledons. The concentrations used on experiments with mung beans were below this level.

The activity of exogenous GA_3 was interfered with by the

addition of B9 and there was evidence that a 100 mg/l it inhibited chlorophyll synthesis when added alone. This supports the possibility that endogenous gibberellins play some part in controlling chlorophyll synthesis and that B9 interferes with GA_3 activity and not its synthesis. At low concentrations of B9, however, the chlorophyll level was increased. This effect casts doubt on the interpretation of the results from the use of B9. Previous reports (Knypl, 1969, 1970) have recorded that B9 inhibited chlorophyll synthesis in cotyledons. The concentrations of B9 used in Knypl's experiments were much higher and, as a result, there was no indication of a stimulatory effect. As in mung bean leaves, the activity of GA_3 in promoting chlorophyll synthesis in cucumber cotyledons was inhibited by B9. The results using growth retardants B9 and CCC are inconclusive but combined with the age/hormone response and endogenous gibberellin assay they indicate that endogenous hormones may play a part in controlling chloroplast development. There is evidence that chloroplasts contain gibberellin-like activity (Stoddart, 1968) which is of a different qualitative pattern from that of the total leaf extract. There is considerable scope in this field of chloroplast research. The evaluation of gibberellin activity of total leaf and chloroplast fractions during greening would yield data having an important bearing on experiments involving their application. It could also lead to the identification of the chloroplast hormones and possibly their use in application experiments.

9. Relationship of Cotyledon, Hypocotyl and Leaf

During primary leaf development the cotyledon performs an essential role in promoting leaf development and chlorophyll synthesis. It was very effective at increasing growth during the period prior to six days and less so after this, while its effect on chlorophyll synthesis appeared to decrease much less rapidly. The reserve of substrate was undoubtedly a major influence in this, however, sucrose was unable to replace the cotyledon, both in its quantitative and qualitative effects. The addition of GA_3 improved the expansion of the leaves and increased the chlorophyll level after 48 hours greening, but this was well below the increase due to the cotyledon. On a weight basis, however, the treatments were almost equally effective. Bean cotyledons are known to contain gibberellins (Wheeler, 1960; Crozier and Audus, 1968; Dale, 1969) and these may be exported with substrate to the leaves. Alternatively, the leaves may manufacture their own gibberellin, (see Results - Section 3), and thus the hormones are active when the cotyledonary substrate arrives. Isolated chloroplasts have been demonstrated to synthesize gibberellins from radioactive kaurenic acid (Stoddart, 1969). The discrepancy between total leaf chlorophyll of the cotyledon and GA_3/sucrose treatments may be explained by the absence of mineral nutrients. Dale (1966) observed that the largest rates of leaf disc expansion occurred in discs treated with carbohydrate nutrients GA_3 and NAA. The effect of the nutrients was about 30% of the sucrose effect. In the mung bean system, the presence of auxin may be assumed, since the primary leaf pair possessing the apical

bud was used as the experimental system. Addition of indoleacetic acid (IAA) was observed to inhibit the level of chlorophyll in these leaves.

The inhibitory effects obtained at some concentrations of sucrose were probably a feature of the method of application. Substrate from the cotyledon would be supplied through the vascular system and only as required by the leaf. In these experiments substrates may have entered through the leaf surface.

The hypotcotyl was also a very effective promoter of chlorophyll synthesis in five day old leaves and on a chlorophyll concentration basis was more effective than the cotyledons. The content per leaf was increased to a similar level by both organs. Since the cotyledon supplies the hypocotyl with substrate and other factors it may be assumed that a proportion of the hypocotyl effect was derived from the cotyledon, but its greater effectiveness may have been due to other factors derived from the root. It is unlikely to be entirely contributed by the cotyledons, because prior to this the cotyledons had a much greater effect on expansion than on the chlorophyll level. When both the hypocotyl and cotyledon were present, the chlorophyll content per leaf was increased to a lesser extent than the sum of the individual effects and chlorophyll concentration was increased only slightly more than by the hypocotyl alone. The effect on dry weight, however, was additive. It may be concluded that the cotyledon and hypocotyl mutually interfere with one another in controlling leaf chlorophyll synthesis, but not in controlling dry weight development. It is conceivable that the

level of substrate supplied by each organ was not at saturation level for dry weight increase but was approaching this for the chlorophyll system. This was supported by the data from Figures 14 and 16 which show that one cotyledon was as effective as two at five days and that lower concentrations of sucrose were more effective than higher ones. Additionally an effect involving hormone balance may have been superimposed. The roots are a possible source of cytokinins (Kulaeva, 1962; Wheeler, 1971), and these must pass through the hypocotyl to reach the leaf. The concentration relationship of the cytokinins from the root, gibberellins in the leaf and substrate derived from the cotyledon and hypocotyl may have resulted in the interference observed between the latter two organs. Further observations of their interaction with age would determine the manner in which this changed and could be linked to a study of the endogenous hormones.

In *P. vulgaris* the opening of the hypocotyl hook is stimulated by GA_3 and inhibited by kinetin in the light and dark (Powell and Morgan, 1970). The cotyledons, however, were inhibitory and the lower hypocotyl had little effect at all. These results suggested the presence of transported "factors", but indicated a system which responded in a different way from chlorophyll synthesis. Reports on the effect of the cotyledon on chlorophyll synthesis in bean primary leaves have been discussed previously. Some direct evidence of inter-organ control of chlorophyll accumulation has recently been reported by De Greef and Caubergs (1972). They found that pre-illumination of the embryonic axis in intact bean plants eliminated the lag phase observed in the leaf, thus suggesting the

transmission of a light-induced stimulus. In the slightly different system where the cotyledons become the first photosynthetic organ, Moore and Lovell (1970) found that the removal of the embryonic axis enhanced chlorophyll production in mustard cotyledons but decreased their photosynthetic rate. They concluded that the embryonic axis competed for material involved in chlorophyll formation, but stimulated the development of other components of the photosynthetic system. The absence of roots as a source of cytokinin may have reduced the photosynthetic activity of the cotyledon (Feierabend, 1969). Hardy, Castelfranco and Rebeiz (1970) demonstrated that the presence of the hypocotyl during illumination enhanced chlorophyll production of cucumber cotyledons during the lag phase. This was a much shorter time period than that of Moore and Lovell (1970) and the material which remained attached to the cotyledons differed slightly. Hardy *et al* (1970) suggested that the hypocotyl "factor" was unlikely to be a general organic nutrient since most of the reserves would be in the cotyledon. None of the classical hormones was able to elicit a response, but ethylene was found to be effective in promoting chlorophyll synthesis (Hardy *et al*, 1971). It is however, possible that part of the nutrients exported to the hypocotyl may be returned to the cotyledon once the hypocotyl has achieved an appropriate length. This may then be utilized in the more essential development of the cotyledon into a photosynthetic organ. Dark growth of the mung bean stem was entirely dependent on the hypocotyl supply (Figure 7).

To obtain a more comprehensive understanding of the role of hormones and organs in controlling mung bean leaf chlorophyll

synthesis, the effect of exogenous substrates and hormones on the leaf in the presence of other organs could be investigated. For example, feeding these substances through the hypocotyl and stem would provide a more normal method of supply to the leaf. This does, however, introduce problems of hormone-induced redistribution of substrate, which may mask direct effects on the leaf.

It may be concluded that chloroplast development in mung bean primary leaves is partially controlled by a complex interaction of translocated factors from the hypocotyl and cotyledons and the nutrient and hormone status of the leaf. Application of gibberellins and cytokinins showed that these hormones may be active in this inter-relationship and that their activity was dependent on photosynthetic development and substrate supply.

The control of chloroplast development by hormones and factors produced at remote sites is an area of research which is still in its infancy. The understanding of the means of chloroplast control is of great importance to our knowledge of photosynthesis and may provide information for improving the available plants. Such systems as the etiolated mung bean leaf could play an important part in solving some of the mysteries.

REFERENCES

REFERENCES

ADEDIPE, N.O., HUNT, L.A. and FLETCHER, R.A. (1971): Effects of benzyladenine on photosynthesis, growth and senescence of the bean plant. Plant Physiol. 25 : 151-153.

AKAZAWA, T. (1965): Starch, inulin and other reserve polysaccharides. in Plant Biochemistry. Ed. J. Bonner and J.E. Varner. Acad. Press 258-297.

AKOYUNOGLOU, G. (1970): The effect of age on the phytochrome-mediated chlorophyll formation in dark-grown bean leaves. Physiol. Plant. 23 : 29-37.

AKOYUNOGLOU, G. and ARGYROUDI-AKOYUNOGLOU, J.H. (1969): Effects of intermittent and continuous light on the chlorophyll formation in etiolated plants at various stages. Physiol. Plant. 22 : 288-295.

AKOYUNOGLOU, G. and ARGYROUDI-AKOYUNOGLOU, J.H. (1971): Photoinduced changes in young bean plants. I. Formation of photosystems I and II. Int. Cong. on Photos. Res. Stresa, 1971. Abstracts p.99.

AKOYUNOGLOU, G., ARGYROUDI-AKOYUNOGLOU, J.H., MICHEL-WOLWERTZ, M.R. and SIRONVAL, C. (1966): Effect of intermittent and continuous light on chlorophyll formation in etiolated plants. Physiol. Plant. 19 : 1101-1104.

AKOYUNOGLOU, G. and MICHALOPOULOS, G. (1971): The relation between the phytylation and the 682 → 672 nm shift *in vivo* of chlorophyll *a*. Physiol. Plant. 25 : 324-329.

ANDERSON, J.M. and BOARDMAN, N.K. (1964): Studies on the greening of dark-grown bean plants II. Development of photochemical activity. Amer. J. Biol. Sci. 17 : 93-101.

ANDERSON, I.C. and ROBERTSON, D.S. (1960): Role of carotenoids in protecting chlorophyll from photodestruction. Plant Physiol. 35 : 531-534.

ARNON, D.I. (1949): Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24 : 1-15.

ARTOMONOV, V.I. (1966): On the synthesis and decomposition of chlorophyll in plants under the influence of gibberellin and vitamin B_2. Fiziol. Rast. 13 : 424-428.

AUGUSTINUSSEN, E. (1964): On the formation of chlorophyll *a* and chlorophyll *b* in etiolated leaves. Physiol. Plant 17 : 403-406.

AUGUSTINUSSEN, E. and MADSEN, A. (1965): Regeneration of protochlorophyll in etiolated barley seedlings following different light treatments. Physiol. Plant. 18 : 828-837.

BANERJII, D. and LALORAYA, M.M. (1967): Chlorophyll formation in isolated pumpkin cotyledons in the presence of kinetin and chloramphenicol. Plant Cell Physiol.8 : 263-268.

BEEVERS, L. (1966): Effect of gibberellic acid on the senescence of leaf discs of nasturtium *(Trapaeolum majus)*. Plant Physiol. 41 : 1074-1076.

BEEVERS, L., LOVEYS, B., PEARSON, J.A. and WAREING, P.F. (1970): Phytochrome and hormonal control of expansion and greening of etiolated wheat leaves. Planta (Berl.) 90 : 286-294.

BENEDICT, C.R. and KOHEL, R.J. (1969): The synthesis of ribulose-1,5-diphosphate carboxylase and chlorophyll in virescent cotton leaves. Plant Physiol. 44 : 621-622.

BERRIDGE, M.V. and RALPH, R.K. (1971): Kinetin and carbohydrate metabolism in Chinese cabbage. Plant Physiol. 47 : 562-567.

BERRY, D.R. and SMITH, H. (1970): The inhibition by high concentrations of (2-chloroethyl)-trimethylammonium chloride (CCC) of chlorophyll and protein synthesis in excised barley leaf sections. Planta (Berl.) 91 : 80-86.

BISHOP, D.E. and SMILLIE, R.M. (1970): The effect of chloramphenicol and cyclohexamide on lipid synthesis during chloroplast development in *Euglena gracilis*. Arch. Biochem. Biophys. 137 : 179-189.

BISHOP, P. and WHITTINGHAM, C.P. (1961): Gibberellic acid and chlorophyll content of leaves and Meteor peas. Nature 192 : 576-577.

BLAAW-JANSEN, G., KOMEN, J.G. and THOMAS, J.B. (1950): On the relation between the formation of assimilatory pigments and the rate of photosynthesis in etiolated oat seedlings. Biochim. Biophys. Acta. 5 : 179-185.

BLACK, M. (1968): How light controls plants. New Scientist 40 : 716-717.

BOASSON, R. and LAETSCH, W.M. (1969): Changes in chloroplast number per cell during leaf development in spinach. Plant (Berl.) 86 : 186-194.

BOASSON, R., LAETSCH, W.M. and PRICE, I. (1972): The etioplast-chloroplast transformation in tobacco: correlation of ultrastructure, replication and chlorophyll synthesis. Amer. J. Bot. 59 : 217-223.

BOGORAD, L. and JACOBSEN, A.B. (1964): Inhibition of etiolated leaves by actinomycin D. Biochem. Biophys. Res. Comm. 14 : 113-117.

BONNER, B.A. (1969): A short-lived intermediate form in the *in vivo* conversion of protochlorophyllide 650 to chlorophyllide 684. Plant Physiol. 44 : 739-747.

BOOTHBY, D. and WRIGHT, S.T.C. (1962): Effects of kinetin and other plant growth regulators on starch degradation. Nature 196 : 389-390.

BOTTOMLEY, W. (1970): Deoxyribonucleic acid-dependent ribonucleic acid polymerase activity of nuclei and plastids from etiolated peas and their response to red and far red light *in vivo*. Plant Physiol. 45 : 608-611.

BRADBEER, J.W. (1969): The activities of the photosynthetic carbon cycle enzymes of greening bean leaves. New Phytol. 68 : 233-245.

BRADBEER, J.W. (1971): Plastid development in primary leaves of *Phaseoulus vulgaris*. The effects of red, far-red and white light treatments on dark-grown plants. J. Exp. Bot. 22 : 382-390.

BRAWERMAN, G. and CHARGAFF, E. (1959): Changes in protein and ribonucleic acid during the formation of chloroplasts in *Euglena gracilis*. Biochim. Biophys. Acta 31 : 164-171.

BRIGGS, G.E. (1920): Experimental researches on vegetable assimilation and respiration XIII. The development of photosynthetic activity during germination. Proc. Roy. Soc. Lond. 91B : 249-268.

BROUGHTON, W.J., HELLMUTH, E.O. and YEUNG, D. (1970): Role of glucose in development of the gibberellin response in peas. Biochim. Biophys. Acta 222 : 491-500.

BROUGHTON, W.J. and McCOMB, A.J. (1971): Changes in the pattern of enzyme development in gibberellin-treated pea internodes. Ann. Bot. 35 : 213-228.

BUSH, L.P. (1969): Influence of certain cations on activity of succinyl CoA synthetase from tobacco. Plant Physiol. 44 : 347-350.

BUTLER, W.L. (1961): Chloroplast development : energy transfer and structure. Arch. Biochem. Biophys. 92 : 287-295.

BUTLER, W.L. (1965): Development of photosynthetic systems 1 and 2 in greening leaf. Biochim. Biophys. Acta 102 : 1-8.

BUTLER, W.L. and BRIGGS, W.R. (1966): The relation between structure and pigment during the first stages of proplastid greening. Biochim. Biophys. Acta 112 : 45-53.

CARRELL, E.F. and KAHN, J.S. (1964): Synthesis of porphyrins by isolated chloroplasts of *Euglena*. Arch. Biochem. Biophys. 108 : 1-6.

CATALANO, M. and HILL, T.A. (1969): Interaction between gibberellic acid and kinetin in overcoming apical dominance, natural and induced by IAA in tomato *Lycopersicium esculentum* Mill. cultivar Potentate. Nature 221 : 985-986.

CHEN, S., McMAHON, D. and BOGORAD, L. (1967): Early effects of illumination on the activity of some photosynthetic enzymes. Plant Physiol. 42 : 1-5.

CHIBNALL, A.C. (1954): Protein metabolism in rooted runner-bean leaves. New Phytol. 53 : 31-37.

CHIN, T-Y. and BEEVERS, L. (1970): Changes in endogenous growth regulators in Nasturtium leaves during senescence. Planta (Berl.) 92 : 178-188.

COHEN, R.Z. and GOODWIN, T.W. (1962): The effect of red and far-red light on carotenoid synthesis by etiolated maize seedlings. Phytochem. 1 : 67-72.

CROMBIE, W.M. (1958): Fatty acids in chloroplasts and leaves. J. Exp. Bot. 9 : 254-261.

CROZIER, A. and AUDUS, L.J. (1968): Distribution of gibberellin-like substances in light- and dark-grown seedlings of *Phaseoulus multiflorus*. Planta (Berl.) 83 : 207-217.

DALE, J.E. (1964): Leaf growth in *Phaseoulus vulgaris* I. Growth of the first pair of leaves under controlled conditions. Ann. Bot. N.S. 28 : 579-590.

DALE, J.E. (1966): The effect of nutritional factors and certain growth substances on the growth of disks cut from young leaves of *Phaseoulus*. Physiol. Plant. 19 : 385-396.

DALE, J.E. (1969): Gibberellins and early growth in seedlings of *Phaseoulus vulgaris*. Planta (Berl.) 89 : 155-164.

DASSIOU, C. and AKOYUNOGLOU, G. (1969): Effect of green and white flashes on the activity of the enzyme ribulose diphosphate carboxylase in dark grown barley plants. Physiol. Plant. 22 : 570-574.

DE DEKEN-GRENSON, M. (1954): Grana formation and synthesis of chloroplastic protein induced by light in portions of etiolated leaves. Biochim. Biophys. Acta 14 : 203-211.

DE GREEF, J.A. and CAUBERGS, R. (1972): Studies on greening of etiolated seedlings. I. Elimination of the lag phase of chlorophyll biosynthesis by a pre-illumination of the embryonic axis in intact plants. Physiol. Plant. 26 : 157-165.

DELMER, D.P. (1972): The regulatory properties of purified *Phaseoulus aureus* sucrose synthetase. Plant Physiol. 47 : 705-708.

DODGE, A.D., ALEXANDER, D.J. and BLACKWOOD, G.C. (1971) : The contribution of photosynthesis to chlorophyll formation in etiolated Mung bean leaves. *Physiol. Plant.* 25 : 71-74.

DODGE, A.D. and WHITTINGHAM, C.P. (1966): Photochemical activity of chloroplasts isolated from etiolated plants. Ann. Bot. 30 : 711-719.

DOWDELL, R.J. and DODGE, A.D. (1970): The photosynthetic capacity of pea leaves with a controlled chlorophyll formation. Planta (Berl.) 94 : 282-290.

DOWDELL, R.J. and DODGE, A.D. (1971): Chlorophyll formation and the development of photosynthesis in illuminated etiolated pea leaves. Planta (Berl.) 98 : 11-19.

EASTWOOD, D., TAVERNER, R.J.A. and LAIDMAN, D.L. (1969): Sequential action of cytokinin and gibberellic acid in wheat aleurone tissue. Nature 221 : 1267.

EDELMAN, J. and HANSON, A.D. (1971): Sucrose supression of chlorophyll synthesis in carrot-tissue cultures. Planta (Berl.) 98 : 150-156.

EILAM, Y. and KLEIN, S. (1962): The effect of light intensity and sucrose feeding on the fine structure in chloroplasts and on chlorophyll content of etiolated leaves. Plant Cell Physiol. 7 : 115-123.

ERIKSSON, G., KAHN, A., WALLES, B. and von WETTSTEIN, D. (1961): Zur makromolekularen physiologie der Chloroplasten III. Ber. deut. bot. Ges. 74 : 221-232.

FALK, R.H. and BOGORAD, L. (1967): Immunological distinction between fraction 1 protein and protochlorophyllide holochrome. Plant Physiol. 44 : 1669-1671.

FEIERABEND, J. (1969): Der einfluss von cytokinen auf die bildung von photosyntheseenzymen in Roggenkeimlingen. Planta (Berl.) 84 : 11-29.

FEIERABEND, J. (1970): Characterization of cytokinin action on enzyme formation during the development of the photosynthetic apparatus in Rye seedlings. Enzymes of the reductive and oxidative pentose phosphate cycles. Planta (Berl.) 94 : 1 - 15.

FELIPPE, G.M. and DALE, G.E. (1968): Effect of a growth retardant, CCC, on leaf growth in *Phaseoulus vulgaris*. Planta (Berl.) 80 : 328-343.

FILNER, B. and KLEIN, A.O. (1968): Changes in enzymatic activities in etiolated bean seedling leaves after a brief illumination Plant Physiol. 43 : 1587-1596.

FLETCHER, R.A. and McCULLAGH, D. (1971): Cytokinin-induced chlorophyll formation in cucumber cotyledons. Planta (Berl.) 101 : 88-90.

FLETCHER, R.A. and OSBORNE, D.J. (1966): Gibberellin as a regulator of protein synthesis and ribonucleic acid synthesis during senescence in leaf cells of *Taraxacum officinale*. Can. J. Bot. 44 : 739-745.

FONDEVILLE, J.C., BORTHWICK, H.A. and HENDRICKS, S.B. (1966): Leaflet movement of *Mimosa pudica* L. indicative of phytochrome action. Planta (Berl.) 69 : 357-364.

FRANK, S.R. (1946): The effectiveness of the spectrum in chlorophyll formation. J. Gen. Physiol. 29 : 157-179.

GABRIELSEN, E.K., MADSEN, A. and VEJLBY, K. (1961): Induction of photosynthesis in etiolated leaves. Physiol. Plant. 14 : 98-110.

GASSMAN, M. and BOGORAD, L. (1967): Control of chlorophyll production in rapidly greening bean leaves. Plant Physiol. 42 : 774-780.

GASSMAN, M., GRANICK, S. and MAUZERALL, D. (1968): A rapid spectral change in etiolated Red Kidney bean leaves following transformation of protochlorophyll. Biochem. Biophys. Res. Comm. 32 : 295-300.

GILES, K.L. (1971): The control of chloroplast division in *Funaria hygrometrica* II. The effects of kinetin and indole-acetic acid on nucleic acids. Plant Cell Physiol. 12 : 447-450.

GIVAN, C.V. and LEECH, R.M. (1971): Biochemical autonomy of higher plant chloroplasts and their synthesis of small molecules. Biol. Rev. 46 : 409-428.

GOODENOUGH, U.W., TOGASAKI, R.K., PASZEWSKI, A. and LEVINE, R.P. (1971): Inhibition of chloroplast ribosome formation by gene mutation in *Chlamydomonas reinhardi*. In Autonomy and Biogenesis of Mitochondria and Chloroplasts. 224-234. Ed. Boardman, Linnane and Smillie. North-Holland.

GOODWIN, R.H. and OWENS, O.H. (1947): The formation of chlorophyll *a* in etiolated out seedlings. Plant Physiol. 22 : 197-200.

GOODWIN, T.W. (1958a): Incorporation of $^{14}CO_2$ [$2^{-14}C$] acetate and [$2^{-14}C$] mevalonic acid into β-carotene in etiolated maize seedlings. Biochem. J. 68 :26p-27p.

GOODWIN, T.W. (1958b): Studies in carotogenesis. 25. The incorporation of $^{14}CO_2$, [$2^{-14}C$] acetate and [$2^{-14}C$] mevalonate into β-carotene by illuminated etiolated maize seedlings Biochem. J. 70 : 612-617.

GRANICK, S. (1963): The pigments of the biosynthetic chain of chlorophyll and their interaction with light. Proc. Vth Int. Congr. Biochem. Moscow. Pergamon Press. 176-186.

GRANICK, S. (1967): The heme and chlorophyll biosynthetic chain. In Biochemistry of Chloroplasts. Ed. T.W. Goodwin. Academic Press. London and New York. II. 373-410.

GREBENSKII, S.O. and PALANITSA, P.V. (1970): Effect of gibberellin on chlorophyll content in leaves and chloroplasts. Fiziol. Rast. 17 : 175-176.

GUNNING, B.E.S. and JAGOE, M.P. (1967): The prolammellar body. In Biochemistry of Chloroplasts. Ed. T.W. Goodwin. Academic Press. London and New York. II. 655-676.

GYLDENHOLM, A.O. (1968): Macromolecular physiology of plastids. V. On the nucleic acid metabolism during chloroplast development. Heriditas 59 : 142-168.

GYLDENHOLM, A.O. and WHATLEY, F.R. (1968): The onset of photophosphorylation in chloroplasts isolated from developing bean leaves. New Phytol. 67 : 461-468.

HALL, D.O. and EVANS, M.C.W. (1972): Photosynthetic phosphorylation in chloroplasts. Sub-Cell. Biochem. 1 : 197-206.

HAMBURG, K.Z. (1964): Growth regulators and plant growth. Quoted in Grebenskii and Palanitsa (1970).

HARADA, H. and LANG, A. (1965): Effect of some (2-chloroethyl) trimethyl-ammonium chloride analogs and other growth retardants on gibberellin biosynthesis in *Fusarium moniliforme*. Plant Physiol. 40 : 176-183.

HARDY, S.I., CASTELFRANCO, P.A. and REBEIZ, C.A. (1970): Effect of the hypocotyl hook on greening in etiolated cucumber cotyledons. Plant Physiol. 46 : 705-707.

HARDY, S.I., CASTELFRANCO, P.A. and REBEIZ, C.A. (1971): Effect of the hypocotyl hook on chlorophyll accumulation in excised cotyledons of *Cucumis sativa* L. Plant Physiol. 47 : 705-708.

HARRIS, J.B. and NAYLOR, A.W. (1968): Changes in the etiolated tobacco leaf during greening.III. The effect of organic metabolites and light intensity on chlorophyll and carotenoid production. Tobacco Sci. XII 171-176. Tobacco 167 : 24-29.

HEDLEY, C.L. and STODDART, J.L. (1971a): Light-stimulation of alanine amino-transferase activity in dark-grown leaves of *Lolium tementulum* L. as related to chlorophyll formation. Planta (Berl.) 100 : 309-324.

HEDLEY, C.L. and STODDART, J.L. (1971b): Factors influencing alanine amino-transferase activity in leaves of *Lolium tementulum* L. II. Effects of growth regulators and protein biosynthesis inhibitors. J. Exp. Bot. 22 : 249-261.

HENDRICKS, S.B. and BORTHWICK, H.A. (1967): The function of phytochrome in regulation of plant growth. Proc. U.S. Nat. Acad. Sci. 58 : 2125-2130.

HENNINGSEN, K.W. (1967): An Action spectrum for vesicle dispersal in bean plastids. In Biochemistry of Chloroplasts. Ed. T.W. Goodwin. Academic Press. London and New York. II. 453-460.

HENNINGSEN, K.W. and BOYNTON, J.E. (1970): Macromolecular physiology of plastids VIII. Pigment and membrane formation in plastids of barley greening under low light intensity. J. Cell Biol. 44 : 290-304.

HILLMAN, W.S. (1957): Non photosynthetic light requirement in *Lemna minor* and its partial satisfaction by kinetin. Science 126 : 165-166.

HUFFAKER, R.C., COX, E.L., KLEINKOPF, G.E. and STANFORD, E.H. (1970): Regulation of synthesis of chlorophyll, carotene, ribulose-1,5 diphosphate carboxylase and phosphoribulokinase in a temperature-sensitive chlorophyll mutant of *Medigago sativa.* Physiol. Plant. 23 : 404-411.

HUFFAKER, R.C., OBENDORF, R.L., KELLER, C.J. and KLEINKOPF, G.E. (1966): Effects of light intensity on photosynthetic carboxylase phase enzymes and chlorophyll synthesis in greening leaves of *Hordeum vulgare* L. Plant Physiol. 41 : 913-918.

HUMPHRIES, E.C. (1958): Effect of gibberellic acid and kinetin on growth in the primary leaves of dwarf bean *(Phaseolus vulgaris)*. Nature 181 : 1081-1082.

HUMPHRIES, E.C. (1963): Effect of 2-chloroethyl trimethyl-ammonium chloride on plant growth, leaf area and net assimilation rate. Ann. Bot. 27 : 517-532.

HUMPHRIES, E.C. (1968): The effect of growth regulators, CCC and B9 on protein and total nitrogen of bean leaves during development. Ann. Bot. 32 : 497-507.

HUMPHRIES, E.C. and FRENCH, S.A.W. (1969): Photosynthesis in sugar beet depends on root growth. Planta(Berl.) 88 : 87-90.

HUMPHRIES, E.C. and WHEELER, A.W. (1960): The effects of kinetin, gibberellic acid and light on expansion and cell division in leaf discs of dwarf bean *(Phaseolus vulgaris)*. J. Exp. Bot. 11 : 81-85.

IRELAND, H.M.M. and BRADBEER, J.W. (1971): Plastid development in primary leaves of *(Phaseolus vulgaris)*. The effects of D-threo and L-threo chloramphenicol on the light-induced formation of enzymes of the photosynthetic carbon pathway Planta (Berl.) 96 : 254-261.

JACOBSEN, J.V. and VARNER, J.E. (1967): Gibberellic acid induced synthesis of protease by isolated aleurone layers of barley. Plant Physiol. 42 : 1596-1600.

JAGENDORF, A.T. and MARGULIES, M.M. (1960): Inhibition of spinach chloroplast photosynthetic reactions by p-chlorophenyl-1, 1-dimethylurea. Arch. Biochem. Biophys. 90 : 184-195.

JENNINGS, R.C., BROUGHTON, W.J. and McCOMB, A.J. (1972): Effect of kinetin on the phycoerythrin and chlorophyll content of a red alga. Phytochem. 11 : 1937-1943.

JONES, R.A. and KAUFFMAN, P.B. (1971): Regulation of growth in *Avena* stem segments by gibberellic acid and kinetin. Physiol. Plant. 24 : 491-497.

JONES, T.W.A. and STODDART, J.L. (1970): Gibberellin-induced changes in protein synthesis and enzyme activity in short apices of *Trifolium pratense*. J. Exp. Bot. 21 : 452-461.

KANNANGARA, C.G., HENNINGSEN, K.W., STUMPF, P.K., APPELQVIST, L. and von WETTSTEIN, D. (1971): Lipid biosynthesis in isolated barley chloroplasts in relation to plastid development. Plant Physiol. 48 : 526-531.

KARABANOV, I.A. (1968): Effect of gibberellin on chlorophyll content in plants. Fiziol. Rast. 15 : 1068-1070.

KARANOV, E. and VASSILEV, L. (1969): The effect of N-6-benzyl-adenine on the growth and gibberellin content of tall and dwarf peas. C.P. Acad. Bulgare des Sci. 22 : 89-93.

KATSUMI, M. and FUKAHARA, M. (1969): The activity of α-amylase in the shoot and its relation to gibberellin-induced elongation. Physiol. Plant. 22 : 68-75.

KAUFFMAN, S. and ALIVISATOS, S.G.A. (1955): Purification and properties of the phosphorylating enzyme from spinach. J. Biol. Chem. 216 : 141-152.

KAUL, K. and SABKARWAL , P.S. (1971): Effects of sucrose and kinetin on growth and chlorophyll synthesis in tobacco tissue cultures. Plant Physiol. 47 : 691-695.

KENDE, H., NINNEMAN, H. and LANG, A. (1963): Inhibition of GA synthesis in *Fusarium moniliforme* by AMO-1618 and CCC. Naturwiss. 50 : 599-600.

KIRK, J.T.O. (1970): Biochemical aspects of chloroplast development Ann. Rev. Plant Physiol. 21 : 11-42.

KIRK, J.T.O. and TILNEY-BASSETT, R.A.E. (1967): The Plastids. Their chemistry, structure, growth and inheritance. W.H. Freeman and Co. London and San Francisco.

KLEIN, S. and BOGORAD, L. (1964): The structural changes in proplastids during photodestruction of pigments. J. Cell. Biol. 22 : 443-451.

KLEIN, S. and NEUMANN, J. (1966): The greening of etiolated bean leaves and the development of chloroplast fine structure in absence of photosynthesis. Plant Cell. Physiol. 7 : 115-123.

KLEIN, S. and POLJAKOFF-MAYBER, A. (1961): Fine structure and pigment conversion in isolated etiolated proplastids. J. Biochem. Biophys. Cytol. 11 : 433-440.

KNYPL, J.S. (1969): Inhibition of chlorophyll synthesis by growth retardants and coumarin, and its reversal by potassium. Nature 224 : 1025-1026.

KNYPL, J.S. (1970): Inhibition of chlorophyll synthesis by growth retarding chemicals and coumarin in detached cotyledons of pumpkin. Biochem. Physiol. Pflanzen. 161 : 1-13.

KNYPL, J.S. and CHYLINSKA, K.M. (1972): The inhibitory effect of (2-chloroethyl) trimethylammonium chloride on chlorophyll and protein synthesis in lettuce cotyledons and its reversal by pottassium. J. Exp. Bot. 23 : 525-529.

KOEHLER, K.H. (1972): Action of inhibitors of protein and nucleic acid synthesis on light-dependent and kinetin-stimulated betacyanin synthesis. Phytochem. 11 : 127-131.

KÖHLER, D. (1966): Die abhängigkeit der gibberellinproduktion von normalerbsen vom phytochrom-system. Planta (Berl.) 98 : 109-116.

KOSKI, V.M. (1950): Chlorophyll formation in seedlings of *Zea mays* L. Arch. Biochem. Biophys. 29 : 339-343.

KOSKI, V.M., FRENCH, C.S. and SMITH, J.H.C. (1951): The action spectrum for the transformation of protochlorophyll to chlorophyll *a* in normal and albino corn seedlings. Arch. Biochem. Biophys. 31 : 1-17.

KOSKI, V.M. and SMITH, J.H.C. (1948): The isolation and spectral absorption properties of protochlorophyll from etiolated barley seedlings. J. Amer. Chem. Soc. 70 : 3558-3562.

KRASNOVSKY, A.A. (1960): The primary processes of photosynthesis in plants. Ann. Rev. Plant Physiol. 11 : 363-410.

KULAEVA, O.N. (1962): The effect of roots on leaf metabolism in relation to the action of kinetin on leaves. Fiziol. Rast. 9 : 229-239.

KURAISHI, I. (1968): The effect of kinetin on protein level of *Brassica* leaf discs. Physiol. Plant. 21 : 78-83.

LANG, A. (1970): Gibberellins : structure and metabolism. Ann. Rev. Plant Physiol. 21 : 537-570.

LAVEE, S. and MESSER, G. (1969): The effect of growth regulating substances and light on olive callus growth *in vitro*. J. Exp. Bot. 20 : 604-614.

LEE, T.T. (1969): Regulation of starch and sugar levels in tobacco leaves by gibberellic acid. Can. J. Bot. 47 : 1595-1598.

LEECH, R.M., RUMSBY, M.G., THOMSON, W.W. and CROSBY, W. (1971): Lipid changes in plastid differentiation in developing maize leaves. Int. Congr. on Photosynthesis. Stresa. Abstracts. p.30.

LEWIS, L.N., COGGINS, C.W., and GARBER, M.J. (1964): Chlorophyll concentration in the navel orange rind as related to potassium gibberellate, light intensity and time. Am. Soc. Hort. Sci. 84 : 177-180.

LITVIN, F.F. and KRASNOVSKY, A.A. (1957): Intermediate stages of formation of chlorophyll in etiolated leaves as studied by fluorescence spectra. Dokl. Akad. Nauk. S.S.S.R. 117 : 106-109.

LOEFFLER, J.E. (1955): Precursors of protochlorphyll in etiolated barley seedlings. Cam. Inst. Year Book 54 : 159-160.

LOVEYS, B.R. and WAREING, P.F. (1971): The red light controlled production of gibberellin in etiolated wheat leaves. Planta (Berl.) 98 : 109-116.

MARGULIES, M.M. (1964): Effect of chloramphenicol on light-dependent synthesis of proteins and enzymes of leaves and chloroplasts of *Phaseoulus vulgaris*. Plant Physiol. 39 : 579-585.

MARGULIES, M.M. (1966): Effect of chloramphenicol on formation of chloroplast structure and protein during greening of etiolated leaves of *Phaseoulus vulgaris*. Plant Physiol. 41 : 992-1003.

MARGULIES, M.M. (1967): Effect of chloramphenicol on chlorophyll synthesis of bean leaves. Plant Physiol. 42 : 218-220.

McMAHON, D. and BOGORAD, L. (1967): Inhibition of photosynthetic enzyme induction by inhibitors of photosynthesis. Federation Proc. 26 : 807.

MEGO, J.L. and JAGENDORF, A.T. (1961): Effect of light on growth of Black Valentine bean plastids. Biochim. Biophys. Acta 53 : 237-254.

MERCER, E.I. and GOODWIN, T.W. (1962): The incorporation of $[2^{-14}C]$ mevalonic acid and $^{14}CO_2$ into the phytyl side chain of chlorophyll in maize seedlings. Biochem. J. 85 : 13p.

MERCER, E.I. and GOODWIN, T.W. (1963): Incorporation of $[2^{-14}C]$ mevalonic acid into steroids of maize seedlings. Biochem. J. 88 : 46p-47p.

MICHEL-WOLWERTZ, M.R. and SIRONVAL, C. (1971): On the induction of PSII function in greening bean leaves. Int. Congr. on Photos. Res. Stresa, 1971. Abstracts p.97.

MILLER, C.O. (1956): Similarity of some kinetin and red light effects. Plant Physiol. 31 : 318-319.

MITRAKOS, K. (1961): The participation of the red far-red reaction system in chlorophyll metabolism. Physiol. Plant. 14 : 497-503.

MONTEVERDE, N.A. (1893): quoted by Kirk and Tilney-Bassett (1967).

MOORE, K. and LOVELL, P. (1970): Differential effects of the embryonic axis on chlorophyll production and photosynthesis of mustard cotyledons. Planta (Berl.) 93 : 289-294.

MOTHES, K. (1959): Bemerkungen über die physiolegischen voraussetzungen der eweissynthese in isolieten blättern. Colleg. Ges. Physiol. Chem. 10 : 72-81.

MURRAY, A.E. and KLEIN, A.O. (1971): Relationship between photo-convertible and non-photoconvertible protochlorophyllides. Physiol. Plant. 48 : 383-388.

NANDI, D.L. and WAYGOOD, E.R. (1965): Biosynthesis of porphyrins in wheat leaves. I. Succinate : CoA ligase (ADP). Can. J. Biochem. 43: 1605-1614.

NARAIN, A. and LALORAYA, M.M. (1970): Inhibition of chlorophyll synthesis by kinetin in *Cucumis* cotyledons. Plant Cell. Physiol. 11 : 173-175.

NEGBI, M. and RUSHKIN, E. (1966): Inhibition of chlorophyll synthesis by growth retardants and counteraction of the effect by gibberellin. Israel J. Bot. 15 : 17-21.

NEUBERGER, A. and TURNER, J.M. (1963): γ,δ-Dioxovalerate amino-transferase activity in *Rhodopseudomonas spheroides*. Biochim. Biophys. Acta 67 : 342-345.

NINNEMAN, H., ZEEVART, J.A.D., KENDE, H. and LANG, A. (1964): The plant growth retardant CCC as inhibitor of gibberellin biosynthesis in *Fusarium moniliforme*. Planta (Berl.) 61 : 229-235.

OBENDORF, R.L. and HUFFAKER, R.C. (1970): Influence of age and illumination on distribution of several Calvin cycle enzymes in greening barley leaves. Plant Physiol. 45 : 579-582.

OELZE-KAROW, H. and BUTLER, W.L. (1971): The development of photophosphorylation and photosynthesis in greening bean leaves. Plant Physiol. 48 : 621-625.

OH-HAMA, T., SHIHIRA-ISHIKAWA, I. and HASE, E. (1965): Development of photosynthetic activities during the process of chloroplast formation of *Chlorella protothecoides*. Plant Cell Physiol. 6 : 743-760.

OOCHAI, S. and HASE, E. (1970): Studies on chlorophyll formation in *Chlorella protothecoides*. I. Enhancing effects of light and added δ-aminolevulinic acid and suppresive effect of glucose on chlorophyll formation. Plant Cell Physiol. 11 : 663-673.

ÖPIK, H. and SIMON, E.W. (1963): Water content and respiration of bean cotyledons. J. Exp. Bot. 14 : 299-310.

OSBORNE, D.J. (1962): Effect of kinetin on protein and nucleic acid metabolism in *Xanthium* leaves during senescence. Plant Physiol. 37 : 595-602.

OSBORNE, D.J. (1967): Hormonal regulation of leaf senescence in Aspects of the biology of ageing. Ed. H.W. Woodhouse. Symp. Soc. Exp. Bot. XXI. 305-321.

OSBORNE, D.J. and McCALLA, D.R. (1961): Rapid bioassay for kinetin and kinins using senescing leaf tissue. Plant Physiol. 36 : 219-221.

PALEG, L. (1960): Physiological effects of gibberellic acid. I. On carbohydrate metabolism and amylase activity of barley endosperm. Plant. Physiol. 35 : 293-299.

PARTHIER, B. and WOLLGIEHN, R. (1961): Uber den einfluss des kinetins auf den eiweiss - und nubleinsäure stoffwecksel in isolierten Tabakblattern. Ber. Deut. Botan. Ges. 74 : 47-51.

PENNER, D. and WILEY, L. (1972): The influence of atrazine, benzyladenine and the embryonic axis on chlorophyll synthesis in cucumber and squash cotyledons. Physiol. Plant. 26 : 148-151.

PHUNG NHU HUNG, S., REMY, R. and MOYSE, A. (1971): Cytochromes and lamellar protein of greening barley etioplasts. Int. Congr. on photosynthesis. Stresa, Abstracts. p.97.

PLESNICAR, M. and BENDALL, D.S. (1971): The development of photochemical activities during greening of etiolated barley. Int. Congr. on photosynthesis. Stresa, Abstracts. p.96.

POGO, B.G.T. and POGO, A.O. (1965): Inhibition by chloramphenicol of chlorophyll and protein synthesis and growth in *Euglena gracilis*. J. Protozool. 12 : 96-100.

POLLACK, R.W. and DAVIES, P.J. (1970): Kinetic studies on chloroplastic ribosomal RNA and chlorophyll development during greening of pea buds. Phytochem. 9 : 471-476.

POSSINGHAM, J.V. and SAURER, W. (1969): Changes in chloroplast number per cell during leaf development in spinach. Planta (Berl.) 86 : 186-194.

POSSINGHAM, J.V. and SMITH, J.W. (1972): Factors affecting chloroplast replication in spinach. J. Exp. Bot. 23 : 1050-1059.

POULSON, R. and BEEVERS, L. (1970): Nucleic acid metabolism during greening and unrolling of barley leaf segments. Plant Physiol. 46 : 315-319.

POWELL, R.D. and MORGAN, P.W. (1970): Factors involved in the opening of the hypocotyl hook of cotton and beans. Plant Physiol. 45 : 548-552.

POWELL, R.D. and GRIFFITHS, M.M. (1960): Some anatomical effects of kinetin and red light on disks of bean leaves. Plant Physiol. 35 : 273-275.

POWER, J.B. and COCKING, E.C. (1970): Isolation of leaf protoplasts : macromolecule uptake and growth substance response. J. Exp. Bot. 21 : 64-70.

PRICE, L. and KLEIN, W.H. (1961): Red, far-red responses and chlorophyll synthesis. Plant Physiol. 36 : 733-755.

PRINGSHEIM, N. (1874): quoted by Kirk and Tilney-Bassett (1967).

REBEIZ, C.A., ABOU-HAIDAR, M., YAGHI, M. and CASTELFRANCO, P.A. (1970): Porphyrin biosynthesis in cell-free homogenates from higher plants. Plant Physiol. 46 : 543-549.

REBEIZ, C.A., YAGHI, M., ABOU-HAIDAR, M. and CASTELFRANCO, P.A. (1970): Protochlorophyll biosynthesis in cucumber cotyledons. Plant Physiol. 46 : 56-63.

REBEIZ, C.A. and CASTELFRANCO, P.A. (1971): Protochlorophyll biosynthesis in a cell-free system from higher plants. Plant Physiol. 47 : 24-32.

REID, D.M., CLEMENTS, J.B. and CARR, D.J. (1968): Red light induction of gibberellin synthesis in leaves. Nature 217 : 580-582.

RHODES, M.J.C. and YEMM, E.W. (1963): Development of chloroplasts and the synthesis of proteins in leaves. Nature 200 : 1077-1080.

RHODES, M.J.C. and YEMM, E.W. (1966): The development of chloroplasts and photosynthetic activities in young barley leaves. New Phytol. 65 : 331-342.

RICHMOND, A.E. and LANG, A. (1957): Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science 125 : 650-651.

RICHMOND, A.E., SACHS, B. and OSBORNE, D.J. (1971): Chloroplasts, kinetin and protein synthesis. Physiol. Plant. 24 : 176-180.

RÖBBELEN, G. (1956): Über die protochlorophyllreduktion in einer mutante von *Arabidopsis thaliana*. Planta 47 : 532-546.

ROGERS, L.J., SHAH, S.P. and GOODWIN, T.W. (1966a): Intracellular localization of mevalonate-activating enzymes in plant cells. Biochem. J. 99 : 381-388.

ROGERS, L.J., SHAH, S.P. and GOODWIN, T.W. (1966b): Mevalonate-kinase isoenzymes in plant cells. Biochem. J. 100 : 14c-17c.

ROMANKO, E.G., KHEIN, Kh. Ya. and KULAEVA, O.N. (1968): Effect of cytokinin on physiological activity of chloroplasts. Biokhimiya 33 : 547-552.

ROTH-BEJERANO, N. and LIPS, S.H. (1970): Seasonal characteristics of interaction between kinetin and gibberellic acid with regard to level of nitrate reductase activity in tobacco leaves. Israel J. Bot. 19 : 30-36.

RYLSKI, I. and HALEVY, A.H. (1972): The effect of damage to cotyledons or primary leaves on the development of pepper *(Capsicum annum* L.*)* Hort. Sci. 7 : 69-70.

SCHOPFER, P. and SIEGELMAN, H.W. (1969): Purification of protochlorophyllide holochrome. Prog. in Photosynthesis Research. Ed. H. Metzner. II. Plastid pigments, electron transport. 611-617.

SEBANEK, J. (1966): Einfluss des kinetins auf den gehalt endogenogibberelline in den wurzeln dekapitierter erbsenkeimlinge. Naturwiss. 53 : 336-

SESTAK, Z. and ULLMAN, J. (1960): The effect of gibberellic acid on the dynamics of chlorophyll synthesis in etiolated seedlings. Biol. Plant. (Praha) 2 : 43-47.

SEYBOLD, A. and EGLE, K. (1938): Zur chromatographischen methode der blattpigmente. Planta 28 : 114-118.

SHEWRY, P.R., PINFIELD, N.J. and STOBART, A.K. (1971): The effect of 2,4-dichlorophenoxyacetic acid and (2-chloroethyl) trimethylammonium chloride on chlorophyll synthesis in barley leaves. Planta (Berl.) 101 : 352-359.

SHIBATA, K. (1957): Spectroscopic studies on chlorophyll formation in intact plants. J. Biochem. 44 : 147-173.

SHLYK, A.A. (1971): Biosynthesis of chlorophyll *b*. Ann. Rev. Plant Physiol. 22 : 169-184.

SHLYK, A.A. and AVERINA, N.G. (1969): Kinetin stimulation of protochlorophyllide accumulation in normal green leaves in the dark. Dokl. Akad. Nauk. S.S.S.R. 186 : 1209-1212.

SIRONVAL, C., MICHEL-WOLWERTZ, M.R. and MADSEN, A. (1965): On the nature and possible functions of the 673- and 684 mμ forms *in vivo* of chlorophyll. Biochim. Biophys. Acta. 94 : 344-354.

SISLER, E.C. and KLEIN, W.H. (1963): The effect of age and various chemicals on the lag phase of chlorophyll synthesis in dark grown bean seedlings. Physiol. Plant. 16 : 315-322.

SMILLIE, R.M. (1963): Formation and function of soluble proteins in chloroplasts. Can. J. Bot. 41 : 123-154.

SMILLIE, R.M., GRAHAM, D., DWYER, R., GRIEVE, A. and TOBIN, N.F. (1967): Evidence for the synthesis *in vivo* of proteins of the Calvin cycle and of the photosynthetic electron-transfer pathway on chloroplast ribosomes. Biochem. Biophys. Res. Comm. 28 : 604-610.

SMITH, H. (1970): Phytochrome and photomorphogenesis in plants. Nature 227 : 665-668.

SMITH, H., STEWART, G.R. and BERRY, D.R. (1970): The effects of light on plastid ribosomal-RNA and enzymes at different stages of barley etioplast development. Phytochem. 9 : 977-983.

SMITH, J.H.C. (1954): The development of chlorophyll and oxygen-evolving power in etiolated barley leaves when illuminated. Plant. Physiol. 29 : 143-148.

SMITH, J.H.C. (1960): Protochlorophyll transformations. In Comp. Biochem. of Photoreactive Systems. Ed. M.B. Allen. Acad. Press. 325-338.

SMITH, J.H.C. and BENITEZ, A. (1954): The effect of temperature on the conversion of protochlorophyll to chlorophyll *a* in etiolated barley leaves. Plant. Physiol. 29 : 135-143.

SMITH, J.H.C., FRENCH, C.S. and KOSKI, V.M. (1952): The Hill reaction: development of chloroplast activity during greening of etiolated barley leaves. Plant Physiol. 27 : 212-213.

SPENCER, D. and WHITFIELD, P.R. (1967a): Ribonucleic acid synthesizing activity of spinach chloroplasts and nuclei. Arch. Biochem. Biophys. 121 : 336-345.

SPENCER, D. and WHITFIELD, P.R. (1967b): DNA synthesis in isolated chloroplasts. Biochem. Biophys. Res. Comm. 28 : 538-542.

STEER, B.T. and GIBBS, M. (1969): Changes in succinyl CoA synthetase activity in etiolated bean leaves caused by illumination. Plant Physiol. 44 : 775-780.

STERN, A.J., SCHIFF, J. and EPSTEIN, H.T. (1964): Chloroplast development in *Euglena*.V. Pigment biosynthesis, photosynthetic oxygen evolution and carbon dioxide fixation during chloroplast development. Plant Physiol. 39 : 220-226.

STOBART, A.K. and PINFIELD, N.J. (1970): Light-induced changes in succinyl-CoA synthetase in barley seedlings. New Phytol. 69 : 31-35.

STOBART, A.K., SHEWRY, P.R. and THOMAS, D.R. (1972): The effect of kinetin on chlorophyll synthesis in ageing etiolated barley leaves exposed to light. Phytochem. 11 : 571-577.

STODDART, J.L. (1965): Chemical changes in *Lolium temulentium* after treatment with CCC. J. Exp. Bot. 16 : 604-613.

STODDART, J.L. (1968): The association of gibberellin-like activity with the chloroplast fraction of leaf homogenates. Planta (Berl.) 81 : 106-112.

STODDART, J.L. (1969): Incorporation of kaurenic acid into gibberellin by chloroplast preparations of *Brassica oleracea*. Phytochem. 8 : 831-837.

SUGIURA, M. (1963): Promotion of chlorophyll synthesis by kinetin. Bot. Mag. Tokyo 76 : 309-310.

SUGIURA, M., UMEMURA, K. and OOTA, Y. (1962): The effect of kinetin on protein level of tobacco leaf disks. Physiol. Plant. 15 : 457-464.

SUNDEQVIST, C. (1969): Transformation of protochlorophyllide formed from exogenous δ-aminolaevulinic acid in continuous light and flashlight. Physiol. Plant. 22 : 147-156.

SUNDEQVIST, C. (1970): The conversion of protochlorophyllide-636 to protochlorophyllide-650 in leaves treated with δ-amino laevulinic acid. Physiol. Plant. 23 : 412-424.

SZALAI, I. (1968): Gibberellinsäure und chlorophyllgehalt des blattes von *Phaseoulus vulgaris* L. Planta (Berl.) 83 : 161-165.

SZALAI, I. (1969): Relation between the chlorophyll content and paleness of gibberellic acid treated leaves. Physiol. Plant. 22 : 587-593.

TANADA, T. (1968): A rapid photoreversible response of barley root tips in the presence of 3-indoleacetic acid. Proc. U.S. Natl. Acad. Sci. 59 : 376-380.

TEVINI, M. (1971): The formation of phospholipids and glycolipids following illumination of etiolated seedlings. Int. Congr. on Photosynthesis. Stresa. Abstracts. p.29.

THORNE, S.W. (1971a): The greening of etiolated bean leaves. I. The initial photoconversion process. Biochim. Biophys. Acta 226 : 113-127.

THORNE, S.W. (1971b): The greening of etiolated bean leaves. II. Secondary and further photoconversion processes. Biochim. Biophys. Acta 226 : 128-134.

THORNE, S.W. and BOARDMAN, N.K. (1971): Formation of chlorophyll *b* and the fluorescent properties and photochemical activities of isolated plastids from greening pea seedlings. Plant Physiol. 47 : 252-261.

TOLBERT, N.E. and GAILEY, F.B. (1955): Carbon dioxide fixation by etiolated plants after exposure to white light. Plant Physiol. 30 : 491-499.

TREHARNE, K.J. and STODDART, J.L. (1968): Effects of gibberellin on photosynthesis in red clover *(Trifolium pratense* L.) Nature 220 : 457-458.

TREHARNE, K.J., STODDART, J.L. PUGHE, J., PARANJOTHY, K. and WAREING, P.F. (1970): Effects of gibberellin and cytokinins on the activity of photosynthetic enzymes and plastid ribosomal RNA synthesis in *Phaseoulus vulgaris* L. Nature 228 : 129-131.

TRÉMOLIÈRES, A. and LEPAGE, M. (1971): Changes in lipid composition during greening of etiolated pea seedlings. Plant Physiol. 47 : 329-334.

TROWN, P.W. (1965): An improved method for the isolation of carboxydismutase. Probable indentity with fraction I protein and the protein moiety of protochlorophyll holochrome. Biochem. 4 : 908-918.

VARNER, J.E. (1964): Gibberellic acid controlled synthesis of α-amylase in barley endosperm. Plant Physiol. 39 : 413-415.

VIRGIN, H.I. (1955): The conversion of protochlorophyll to chlorophyll *a* in continuous and intermittent light. Physiol. Plant. 8 : 389-403.

VIRGIN, H.I. (1958): Studies on the formation of protochlorophyll and chlorophyll *a* under varying light treatments. Physiol. Plant. 11 : 347-362

VIRGIN, H.I. (1960): Pigment transformation in leaves of wheat after irradiation. Physiol. Plant 13 : 155-164.

VIRGIN, H.I., KAHN, A. and von WETTSTEIN, D. (1963): The physiology of chlorophyll formation in relation to structural changes in chloroplasts. Photochem. Photobiol. 2 : 83-91.

VOROBAEVA,L.M., BYSTROVA, M.I. and KRASNOVSKY, A.A. (1963): Phytolic and non-phytolic forms of pigments in leaves and homogenates. Biokhimiya 28 : 426-433.

VYVYAN, M.C. (1924): Studies on the rate of growth of leaves by a photographic method. I. The determination of the rate of growth of first leaves of *Phaseoulus vulgaris*. Ann. Bot. 38 : 59-103.

WALLACE, J.W. and NEWMAN, D.W. (1965): Lipids of proplastids and nitrogen - deficient chloroplasts. Phytochem. 4 : 43-47

WAREING, P.F., KHALIFA, M.M. and TREHARNE, K.J. (1968): Rate limiting processes in photosynthesis at saturating light intensities. Nature 220 : 453-457.

WELLBURN, F.A.M. and WELLBURN, A.R. (1971): Chlorophyll synthesis by isolated intact etioplasts. Biochem. Biophys. Res. Comm. 45 : 747-750.

von WETTSTEIN, D. (1958): The formation of plastid structures. Brookerhaven Symp. in Biology 11. 138-159.

von WETTSTEIN, D., HENNINGSEN, K.W., BOYNTON, J.E., KANNANGARA, C.G., and NIELSEN, O.F.:(1971): The genic control of chloroplast development in barley. Autonomy and biogenesis of mitochondria and chloroplasts 205-223. Ed. Boardman, Linnane and Smillie. North-Holland.

WHEELER, A.W. (1960): Changes in a leaf-growth substance in cotyledons and primary leaves during growth of dwarf bean seedlings. J. Exp. Bot. 11 : 217-226.

WHEELER, A.W. (1966): Effect of removing cotyledons, apical growing region, or trifoliate leaves on the growth and growth substance content of dwarf French bean *(Phaseoulus vulgaris)*. J. Exp. Bot. 17 : 621-626.

WHEELER, A.W. (1971): Auxins and cytokinins exuded during formation of roots by detached primary leaves and stems of dwarf French beans *(Phaseoulus vulgaris* L.*)* Planta (Berl.) 98 : 128-135.

WHEELER, A.W. and HUMPHRIES, E.C. (1963): Effect of gibberellic acid on growth, gibberellin content and chlorophyll content of leaves of potato *(Solanum tuberosum)*. J. Exp. Bot. 14 : 132-136.

WHYTE, P. and LUCKWILL, L.C. (1966): A sensitive bioassay for gibberellins based on retardation of leaf senescence in *Rumex obtusifolus* L. Nature 210 : 1360.

WITHROW, R.B., WOLFF, J.B. and PRICE, L. (1956): Elimination of the lag phase of chlorophyll synthesis in dark-grown leaves by a pre-treatment with low irradiances of monochrome energy. Plant Physiol. 31 : xiii-xiv.

WITTWER, S.H. and BUKOVAC, M.J. (1958): The effects of gibberellin on economic crops. J. Econ. Bot. 12 : 213-255.

WOLFF, F.T. and HABER, A.H. (1960): Chlorophyll content of gibberellin-treated wheat seedlings. Nature 186 : 217-218.

WOLFF, J.B. and PRICE, L. (1957): Terminal steps of chlorophyll *a* biosynthesis in higher plants. Arch. Biochem. Biophys. 72 : 293-301.

WOLFF, J.B. and PRICE, L. (1960): The effect of sugars on chlorophyll biosynthesis in higher plants. J. Biol. Chem. 235 : 1603-1608.

WOLKEN, J.J. and MELLON, A.D. (1956): The relation between chlorophyll and the carotenoids in the algal flagellate, *Euglena*. J. Gen. Physiol. 39 : 675-685.

ZEEVART, J.A.D. (1966): Reduction of the gibberellin content of *Pharbitis* seeds by CCC and after effects in the progeny. Plant. Physiol. 41 : 856-862.

ZIEGLER, H. and ZIEGLER, I. (1965): Der einfluss der belichtung auf die $NADP^+$-abhängige gylcerinaldehyde-3-phosphate-dehydrogenase. Planta (Berl.) 65 : 369-380.

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The Interaction of a Gibberellin and a Kinin in the Control of Chlorophyll Synthesis

Chlorophyll formation in etiolated plant material is a light requiring process. In darkness the immediate precursor of chlorophyll, protochlorophyll, accumulates and this is photoreduced to chlorophyll. This terminal step is at the end of a complex biosynthetic pathway, and the continuing production of chlorophyll is associated with a parallel development of chloroplast thylakoid structure[1]. Many physiological and biochemical treatments have an apparent effect on chlorophyll formation in a variety of known and unknown ways. A few examples of this are the phytochrome-mediated abolition of the lag phase of chlorophyll synthesis[2–5], the inhibition of chlorophyll formation by a range of antibiotics[6–9], and a range of responses elicited by growth regulators. Gibberellins[10–13] and kinins[14–16] have been reported to promote[10, 11, 14, 15] and inhibit [12, 13, 16] chlorophyll formation in a variety of plant materials.

In this preliminary report we have investigated the effects of a gibberellin (gibberellic acid, GA_3) and a kinin (6-benzyl-aminopurine, 6-BAP) on chlorophyll formation in etiolated mung bean leaves.

The treated plant material consisted of the two primary leaves with petioles and the minute apical bud. These were detached from seedlings which had been grown in total darkness on vermiculite for 5 days at 25 °C. Four uniform samples of 5 leaf pairs for each treatment were placed on Whatman number 3 filter paper in 9 cm crystallizing dishes, containing 10 ml of the appropriate solutions. GA_3 was prepared in aqueous solution and 6-BAP contained 0.05% dimethyl formamide at 10 mg/l. This was used to initially dissolve the cytokinin and did not effect chlorophyll synthesis. After 4 h dark incubation, the leaf pairs were illuminated at 1000 lux for 48 h at 25 °C. Chlorophyll was extracted in 80% acetone and the concentration determined using the method of ARNON[17].

The results in the Table reveal a synergistic promotion of chlorophyll synthesis at 1,5 and 10 mg/l 6-BAP in combination with 0.01, 0.1, 1.0 and 10 mg/l GA_3. A peak for this enhancement occured at 5 mg/l GA_3. Synergistic inhibition of chlorophyll formation was also observed with a maximum effect at 0.01 mg/l 6-BAP in the presence of 0.001 mg/l GA_3. The effect of these hormones on their own was small. A slight promotion was observed with most GA_3 concentrations, while 6-BAP showed an inhibition at the lower concentrations and a promotion at 5 mg/l.

The effect of a gibberellin (GA_3) and a kinin (6-BAP) on chlorophyll formation in etiolated bean leaves

Gibberellic acid GA_3 (mg/l)	6-Benzyl-aminopurine (mg/l) 0	0.001	0.01	0.1	1.0	5.0	10.0
0	100	84	71	94	95	112	105
0.001	110	77	49	87	109	119	11
0.01	110	86	56	85	120	169	131
0.1	103	77	56	105	129	174	120
1.0	107	83	90	100	143	169	117
10	117	94	95	103	126	156	129

The chlorophyll content per leaf is expressed as a percentage of the control level.

The relationship of the concentrations of GA_3 and 6-BAP which produced an optimum promotion showed that as the concentration of 6-BAP was increased, the concentration of GA_3 necessary for this optimum effect decreased. A similar interrelationship between GA_3 and kinetin was found by ROTH-BEJERANO and LIPS[18] for the induction of nitrate reductase activity in summer-grown tobacco. The addition of both hormones to discs of dwarf french bean leaves resulted in a stimulation of expansion, but their action was additive rather than synergistic[19]. KURSANOV et al.[20], however, elicited a synergistic response with 10 mg/l BAP + 1 mg/l GA_3 on the expansion of isolated pumpkin cotyledons.

The results reported above were obtained with leaves detached from 5-day-old seedlings, and these were the maximal responses elicited over a wider age range. It is possible that this maximal effect was related to the internal hormonal and substrate status of the leaves, which was determined, prior to detachment by the physiological interaction of leaves, cotyledons, hypocotyl and roots.

Resumé. La promotion synergétique et l'inhibition de la synthèse de la chlorophylle par la gibberelline (GA_3) et par la 6-benzylaminopurine (6-BAP) de concentrations diverses, fut observée. L'augmentation de la concentration de 6-BAP réduisit la concentration de GA_3 nécessaire pour obtenir la promotion maximum.

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[1] H. I. VIRGIN, A. KHAN and D. VON WETTSTEIN, Photochem. Photobiol *2*, 83 (1963).
[2] R. B. WITHROW, J. B. WOLFF and L. PRICE, Pl. Physiol. *31*, 13 (1956).
[3] H. I. VIRGIN, Physiologia plant. *14*, 439 (1961).
[4] L. PRICE and W. H. KLEIN, Pl. Physiol. *36*, 733 (1961).
[5] K. MITRAKOS, Physiologia plant. *14*, 497 (1961)
[6] M. M. MARGULIES, Pl. Physiol. *37*, 473 (1962).
[7] J. T. O. KIRK and R. L. ALLEN, Biochem. biophys. Res. Commun. *14*, 393 (1965).
[8] L. BOGORAD and A. B. JACOBSON, Biochem. biophys Res. Commun. *14*, 113 (1964).
[9] R. J. DOWDELL and A. D. DODGE, Planta *98*, 11 (1971).
[10] A. W. WHEELER and E. C. HUMPHRIES, J. exp. Bot. *14*, 132 (1963).
[11] M. NEGBI and E. RUSHKIN, Israel J. Bot. *15*, 17 (1966).
[12] I. SZALAI, Planta *83*, 161 (1968).
[13] I. SZALAI, Physiologia plant. *22*, 587 (1969).
[14] D. BANERJI and M. M. LALORAYA, Pl. Cell Physiol. Tokyo. *8*, 263 (1967).
[15] M. SUGIURA, Bot. Mag. Tokyo *76*, 309 (1963).
[16] A. NARAIN and M. M. LALORAYA, Pl. Cell Physiol., Tokyo *11*, 173 (1970).
[17] D. I. ARNON, Pl. Physiol *24*, 1 (1949).
[18] N. ROTH-BEJERANO and H. S. LIPS, Israel J. Bot. *19*, 30 (1970).
[19] E. C. HUMPHRIES and A. W. WHEELER, J. exp. Bot. *11*, 81 (1960).
[20] A. L. KURSANOV, O. N. KULAEVA and T. P. MIKULOVICH, Am. J. Bot. *56*, 767 (1969).